Apolipoprotein E Isoforms Differentially Regulate Matrix Metallopeptidase 9 Function in Alzheimer's Disease

Thesis

PHD THESIS

of

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Apolipoprotein E Isoforms Differentially Regulate Matrix Metallopeptidase 9 Function in Alzheimer's Disease

Discipline
Neuroscience

Date of Submission
July 2020

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Declaration

I hereby declare that the work presented in this thesis is my own, except where stated. This work has not been submitted for any other degree of professional qualification.

Charis Ringland.

Abstract

Apolipoprotein E (APOE) is a major genetic risk factor for Alzheimer's disease (AD) and has been shown to influence amyloid-β (Aβ) clearance from the brain in an isoform-specific manner. Our prior work showed Aβ transit across the blood-brain-barrier was reduced by apoE4, compared to other apoE isoforms, due to elevated lipoprotein receptor shedding in brain endothelia. Recently, we demonstrated matrix metallopeptidase 9 (MMP-9) induces lipoprotein receptor proteolysis in an apoE isoform-dependent manner, which impacts Aβ elimination from the brain. The current studies interrogated the relationship between apoE and MMP-9 and found apoE dose-dependently reduced MMP-9 activity in a cell-free assay, with apoE4 showing a significantly weaker ability to inhibit MMP-9 function than apoE2 or apoE3. Moreover, these effects may be due to the reduced binding affinity of apoE4 for MMP-9 compared to apoE2 and apoE3 as revealed by kinetic binding studies. Elevated MMP-9 expression and activity was observed in the cerebrovasculature of both human and animal AD brain specimens with an APOE4 genotype. The apoE isoforms also lead to altered levels of MMP-9 secreted from brain endothelia cultures (apoE2<apoE3=apoE4). Both the expression and secretion of MMP-9 were more pronounced upon insult, suggesting a combined influence of AD and APOE genotype. Collectively, these findings suggest a role for apoE in regulating MMP-9 disposition in the brain, which could have profound consequences for a variety of neurodegenerative diseases. With respect to AD, genetic deletion of the MMP-9 gene in 5xFAD mice rescued deficits in sociability, social recognition memory and anxiety disinhibition, signifying an important role for MMP-9 in the behavioral dysfunction inherent in AD. These observations did not appear to be due to changes in brain Aβ levels or lipoprotein receptor shedding, suggesting the behavioral improvements resulting from MMP-9 gene removal were mediated through alternative mechanisms. In total, modulating MMP-9 may represent a promising therapeutic strategy for the treatment of AD and other neurological disorders.

Acknowledgements

It is a pleasure to thank the following people, without whom I would not have been able to complete this research.

First of all, I would like to express my sincere gratitude to my supervisor Dr. Corbin Bachmeier for the continuous encouragement and support throughout my Ph.D. study. His guidance greatly facilitated all the planning and conducting of experiments and in the writing of this thesis. I count myself very lucky to have had such a supervisor for my Ph.D.

I wish to express my special appreciation and thanks to my secondary supervisor, Dr. Laila Abdullah, who has provided a great deal of guidance and feedback over the course of my Ph.D. I am exceptionally grateful for her help and expertise with statistical analysis and the apoE lipidation studies.

Thank you both for guiding me through the research and the preparation of both this thesis and the published manuscript.

Of course, I am extremely indebted to Dr. Fiona Crawford and Dr. Mike Mullan for their support of me and all the other Ph.D. students. I am thankful for Mike's comprehensive knowledge and his encouragement for us to take the time to deliberate over decisions and to consider the bigger picture. Thank you to Fiona for always being willing to make time to help and for being an unstoppable force of good for the institute. I would like to say an extra thanks to you both for all the measures you have put in place with regards to the current unprecedented situation caused by COVID-19, to ensure that the Roskamp Institute remains a safe haven in amongst the turmoil.

Thank you to Robert and Diane Roskamp for co-founding the Roskamp Institute and for providing a place for scientists to conduct research with the aim to combat neurological diseases. Thank you to the Roskamp foundation, to the Bay Pines Veteran's Affairs Healthcare system and to the National Institute of Health for funding this research.

I would like to say a big thank you to the other PIs at the Roskamp Institute, in particular Dr. Daniel Paris and Dr. Ghania Ait-Ghezala, for their individual expertise and help in various aspects of my project. I would also like to acknowledge and thank Dr. Ben Shackleton for his prior research which provided the foundation for these studies. Thank you to Max Eisenbaum for lending a hand whenever I needed it and for always being ready with a joke. I would also like to thank the other current and past students and staff that have helped me and who have made my overall experience enjoyable. In particular, Alex Morin, Claire Huguenard, Adam Cseresznye, Heather Langlois and Nicole Saltiel.

My special thanks to my family for their continued encouragement and support of my education and of the choices that I make for my life. Thank you for your visits to the U.S. during my Ph.D. and for always enquiring about my progress.

And finally, thank you to my brilliant husband and fellow Ph.D. student, Dr. Jonas Schweig. You have always been my support from the beginning, happy to share your knowledge and experience and willing to give up your free time to help me solve a problem. Thank you for supporting me both in the laboratory and at home.

I would also like to thank the scientists that actively contributed to this thesis:

The binding analysis of apoE to MMP-9 displayed in Chapter 3.3.1 was performed by Dr. Daniel Paris.

The cell-free activity assay, showing the regulation of MMP-9 activity by apoE, displayed in Chapter 3.3.3, was conducted by Dr. Ben Shackleton.

The confocal analysis of EFAD tissue displayed in Chapter 2.3.2 and Chapter 3.3.4 was conducted by Dr. Jonas Schweig.

The ELISA analysis of lipoprotein receptor levels in human brain tissue displayed in Chapter 3.3.5 were derived from the Ph.D. thesis of Dr. Ben Shackleton.

This work was supported by Merit Review award number I01BX002839 from the United States (U.S.) Department of Veterans Affairs (VA) Biomedical Laboratory Research and Development Program. The research in this publication was also supported by the National Institute on Aging of the National Institutes of Health under award number R01AG041971. I am grateful to the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona for providing the human brain specimens. The Brain and Body Donation Program is supported by the National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson's Disease and Related Disorders), the National Institute on Aging (P30 AG19610 Arizona Alzheimer's Disease Core Center), the Arizona Department of Health Services (contract 211002, Arizona Alzheimer's Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, 05-901 and 1001 to the Arizona Parkinson's Disease Consortium) and the Michael J. Fox Foundation for Parkinson's Research. Human brain tissue specimens were also obtained from the University of Maryland Brain and Tissue Bank which is a Brain and Tissue Repository of the NIH NeuroBioBank (Baltimore, MD) and the Mount Sinai NIH Brain and Tissue Repository (New York, NY). I would also like to thank Dr. Mary Jo LaDu (University of Illinois at Chicago) for providing the mixed glial cultures and EFAD animals, and Dr. Hussein Yassine (University of Southern California) for his guidance and assistance with the apoE lipidation studies.

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Abbreviations

AD	Alzheimer's disease
ADAM10	A desintegrin and metalloproteinase domain containing protein 10
AICD	APP intracellular domain
ANOVA	Analysis of variance
APMA	4-aminophenylmercuric acetate
APOE	Apolipoprotein E
ApoE-TR	ApoE targeted replacement
APP	Amyloid precursor protein
ARIA	Amyloid-related imaging abnormalities
Aβ	Amyloid beta
BACE	β-site amyloid precursor protein cleaving enzyme
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BKY	Two-stage step-up method of Benjamini, Krieger and Yekutieli
C1q	Complement component 1q
CNS	Central nervous system
CSF	Cerebrospinal fluid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EOAD	Early-onset Alzheimer's disease
EPM	Elevated plus maze
FAD	Familial Alzheimer's disease
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
GI	Guanidine insoluble
GS	Guanidine soluble
HBMECs	Human brain microvascular endothelial cells
HBSS	Hanks Balanced Salt Solution
HDL	High-density lipoprotein
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
Ig	Immunoglobulin
IL	Interleukin

KD	Dissociation constant
KO	Knockout
LDLR	Low-density lipoprotein receptor
LOAD	Late-onset Alzheimer's disease
LRP1	Low density lipoprotein receptor-related protein 1
LTP	Long-term potentiation
MAPT	Microtubule associated protein tau
MCI	Mild cognitive impairment
MMP	Matrix metallopeptidase
MMP9KO	MMP-9 knockout
MPER	Mammalian Protein Extraction Reagent
NFTs	Neurofibrillary tangles
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
OFT	Open field test
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PET	Positron emission tomography
PFA	Paraformaldehyde
PKC-α	Protein kinase C-α
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulphonyl fluoride
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PS1	Presenilin 1
PS2	Presenilin 2
RAGE	Receptor for advanced glycation end products
RAWM	Radial arm water maze
SB-3CT	2-[[(4-Phenoxyphenyl)sulfonyl]methyl]thiirane)
SEM	Standard error of the mean
TBI	Traumatic brain injury
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metallopeptidases
TNF-α	Tumour necrosis factor α
TREM2	Triggering receptor expressed on myeloid cells 2
VCAM1	Vascular cell adhesion molecule-1
WT	Wild type
α-CTF	α-C-terminal fragment
β-CTF	β-C-terminal Fragment

Chapter 1: Introduction

1.1 Alzheimer's disease

1.1.1 Incidence and impact of Alzheimer's disease

Dementia is an umbrella term for diseases and conditions characterised by a gradual loss in memory and cognitive ability [1]. Today (2020), it is estimated that there are over 50 million people living dementia. With the rapid growth in the number of individuals developing dementia, this figure is set to triple to 152 million by 2050 [2], largely due to the aging population. Indeed, age is the most influential known risk factor; it is estimated that 25% to 45% of people older than 85 years have some form of dementia, the most common being Alzheimer's disease (AD), accounting for more than half of all cases [1], [3]. However, AD can also develop earlier, with symptoms appearing between the ages of 30 and 60. Based on the age of onset, AD is subdivided into either early-onset AD (EOAD), which is usually linked to causative genes, or the more prevalent form, late-onset AD (LOAD), also referred to as sporadic AD, where the cause is unknown but associated with gene-environment interactions. Typically, EOAD tends to have a more aggressive disease progression and a shorter relative survival time [4].

Neurofibrillary tangles of hyperphosphorylated tau (NFTs), accumulation of amyloid beta (Aβ) and neuroinflammation are the classic hallmark pathologies that characterise AD and were first described by Alois Alzheimer in 1907 [5]. Although the definitive diagnosis of the disease requires post-mortem examination of the brain and the identification of Aβ plaques, diagnosis in living patients can be assisted by certain clinical criteria and cerebrospinal fluid (CSF) and positron emission tomography (PET) biomarkers [6]–[8]. Current validated CSF biomarkers for AD are low Aβ-42 levels, high total tau and high phosphorylated tau protein levels [9], [10]. As it is a progressive and currently irreversible neurodegenerative disorder, symptoms of AD usually develop gradually and worsen over time, becoming debilitating and interfering with daily activities. They progress from mild loss of memory in the early stages of AD to being unable to respond to

the environment and carry on conversations, as is often the case in late-stage AD. The most common early symptom of AD is having problems retaining new information. As the disease develops through the brain, the symptoms become increasingly severe and may manifest as mood and behaviour changes such as confusion about events, time and place or becoming suspicious about family and friends. People with AD may also have difficulty speaking, swallowing and walking [2].

1.1.2 Amyloid pathology

1.1.2.1 APP processing

The amyloid precursor protein (APP), located on chromosome 21, is thus named because it was demonstrated to be the precursor protein to Aβ peptides, which are the building blocks of the extracellular plaques central to AD [11]–[17]. The production of Aβ requires the double cleavage of APP via the amyloidogenic pathway. First, β-secretase, also known as β-site amyloid precursor protein cleaving enzyme (BACE), cleaves APP within the luminal domain generating membrane-tethered β-C-terminal fragment (β-CTF) and the soluble ectodomain sAPPβ [18]. Next, the β-terminal fragments are cleaved by γ-secretase within the transmembrane domain to release Aβ. Both Aβ and sAPPβ are released into the extracellular milieu following exocytosis [19]. Alternatively, APP can also be processed via the nonamyloidogenic pathway which involves α-secretase as opposed to BACE, and cleaves APP within the Aβ sequence, thus preventing its production. In this pathway, γ-secretase then cleaves the α-C-terminal fragments (α-CTF) resulting in the formation of p3 peptides instead of Aβ (Figure 1.1). The accumulation of soluble Aβ and the subsequent aggregation into Aβ plaques seen in AD can be attributed in part to a shift in the equilibrium of the APP processing towards the amyloidogenic pathway [20].

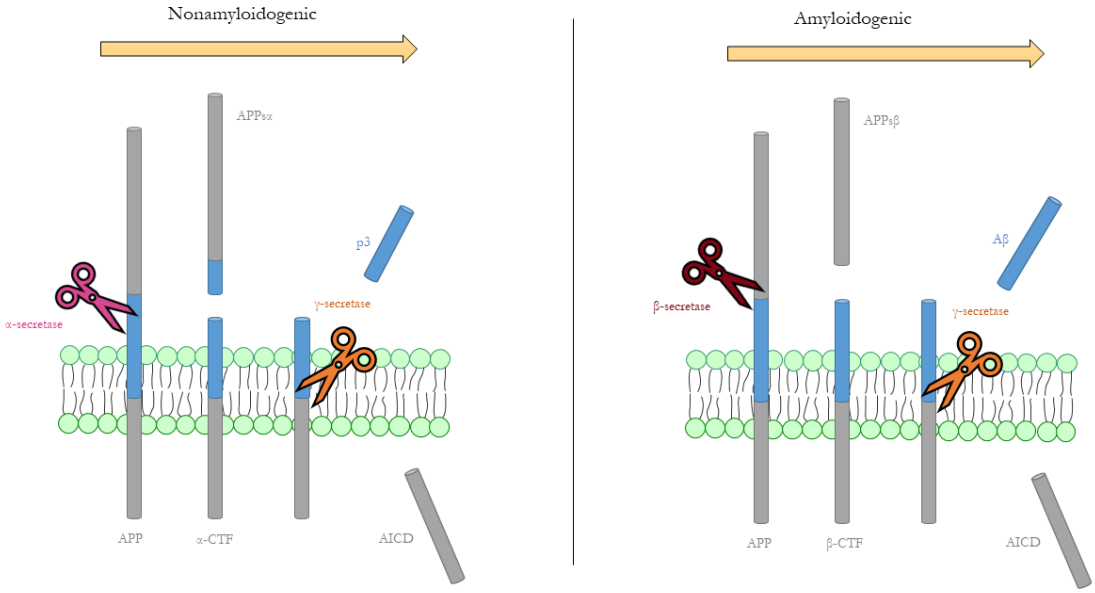


Figure 1.1: Processing of β-amyloid precursor protein by the sequential processing of secretases.
A) Nonamyloidogenic processing of APP by α-secretase followed by γ-secretase releasing the p3 peptide. B) Amyloidogenic processing of APP by β-secretase and subsequently γ-secretase resulting in the production of the Aβ protein. Both α-secretase and β-secretase generate soluble ectodomains (sAPPα and sAPPβ respectively) and in both instances intracellular C-terminal fragments (AICD) are produced following γ-secretase cleavage (as described by O'Brien and Wong (2011) [21]).

1.1.2.2 Mutations in genes involved in APP processing

Epidemiological studies provided the first direct evidence to implicate the considerable involvement of genetic factors in AD pathology. AD can be divided into a familial and sporadic forms based on whether the disease can be traced to a clear family history [22]. While the majority of AD cases are classed as sporadic, the clinical course and pathology of familial AD (FAD) is similar to sporadic AD and thus has been used extensively to study the disease as a whole and to conduct clinical trials (discussed in 1.4). Thus, it is crucial to study and define the molecular mechanisms that give rise to AD in individuals with FAD [23]. Three causative genes have been linked to autosomal dominant FAD: *APP*, *PS1*, and *PS2*. The first mutations of these genes that were found to cause AD were discovered in the early 1990s and include point/missense mutations of the APP protein, e.g. APP V717I (London) and APP KM670/671NL (Swedish) [24], [25]. These mutations were the first of many implicating APP in the development of EOAD [26]. One variant (APP A673T (Icelandic)), uncovered in 1993, was demonstrated not to cause AD but to be protective against the disease and cognitive decline in the elderly without AD [27], [28]. *In vitro*, it was associated with a 40% reduction in the formation of amyloidogenic peptides which was attributed to the mutation being in close proximity to, and inhibiting the cleavage of, BACE [27].

Further mutations that affect the cleavage of APP were detected in genes encoding subunits of the γ-secretase, presenilin 1 and 2 (PS1, PS2) [4], [29]–[35]. When these mutations were identified, PS1 mutations were found to occur more frequently than PS2 mutations and now over 200 different PS1 mutations have been recognised [35], [36], accounting for up to 70% of all EOAD cases [37]. Presenilin gene mutations typically result in the elevated production of Aβ-42, causing a higher ratio of Aβ-42:Aβ-38 and a consequently greater formation of Aβ aggregates [38]–[40].

1.1.2.3 The amyloid hypothesis

Glenner and Wong (1984b) first isolated and sequenced the Aβ protein and proposed that it could be a causative factor in AD [12]. The subsequent discovery that pathogenic mutations of APP,

PS1 and PS2 lead to the aberrant cleavage of APP and hence distort the normal processing of the Aβ protein, has provided impetus to the reigning amyloid cascade hypothesis. This postulates that Aβ is imperative to the pathogenesis of AD and positions it at the beginning of the pathological cascade for the disease process: "beta-amyloid deposition----tau phosphorylation and tangle formation----neuronal death" [41]–[44]. More genetic evidence that supports this hypothesis arises from the finding that the extra copy of chromosome 21 and the ensuing overexpression of the APP gene in individuals with Trisomy 21 (Down's syndrome) results in elevated Aβ deposits which precede further pathology characteristic of AD [45].

Accumulation of Aβ occurs early in the disease pathology, and besides the increased production of Aβ contributing to the increased levels, there is also thought to be an imbalance in the clearance of the peptide (discussed in 1.2). While FAD presents with mutations that encourage the increased production of Aβ, the impaired clearance is deemed to be of more consequence in the sporadic forms of AD, where the failure of clearance mechanisms gives rise to the gradual accrual of the toxic amyloidogenic proteins [45]. Yet, according to the amyloid cascade hypothesis, in both cases the oligomerisation of Aβ affects synaptic health and the deposition of Aβ oligomers into diffuse plaques elicits inflammatory responses and neuronal dyshomeostasis. This leads to changes in kinase and phosphatase activity and the formation of NFTs comprised of the protein tau (discussed in more detail in 1.1.3). Ultimately this leads to extensive neuronal dysfunction and death, resulting in dementia (Figure 1.2) [45].

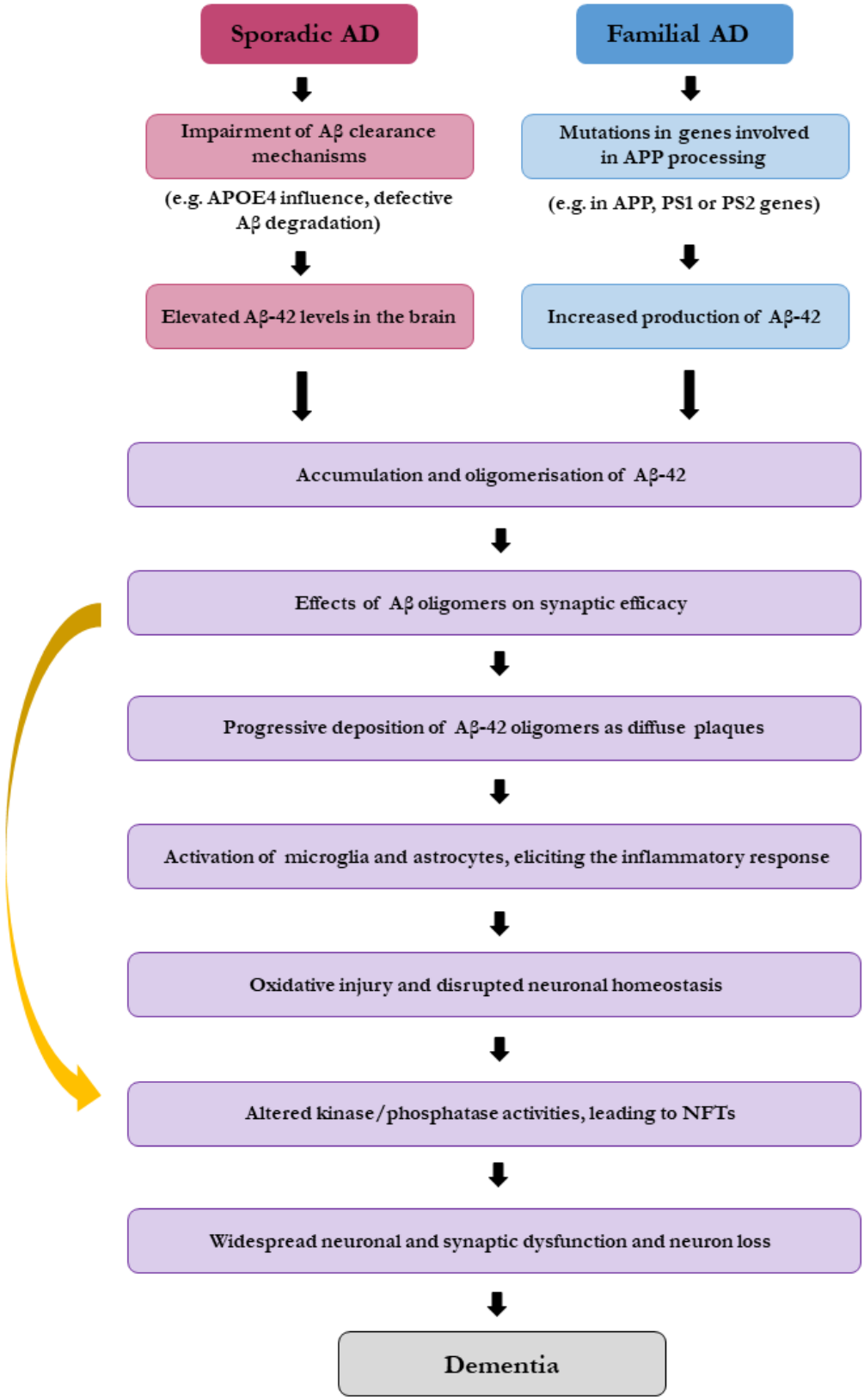


Figure 1.2: The amyloid cascade hypothesis describing the sequence of major pathogenic events leading to AD.
The curved yellow arrow signals that Aβ oligomers may also contribute to downstream processes such as activating microglia and astrocytes and directly injuring synapses and neurons (as described by Selkoe and Hardy (2016) [45]).

Although this linear hypothesis has been met with considerable scepticism since its conception, Aβ still remains entwined in AD pathology and represents a compelling target for treatment of the disorder [46]–[50]. In their reviews 10 and 25 years after, the authors discuss the research that has been conducted since which has supported the hypothesis and provide counterarguments for the findings that highlight discrepancies in it [45], [51]. One such disparity concerns the poor correlation of amyloid plaque burden with the degree of cognitive impairment compared to tau tangles. Indeed, tau has been shown to correlate well with cognitive impairment over the course of AD pathogenesis [52]. Nevertheless, Aβ deposits typically appear very early in the disease pathology and have been shown to lead to the downstream cellular and molecular changes such as neuroinflammation and aggregation of misfolded hyperphosphorylated tau, that are more causal to neuronal dysfunction [45], [53]–[55]. Accumulating research suggests that amyloid is present at the disease onset and precipitates the spread of tau, allowing it to cause further damage to neurons [56]–[59]. This is one of the ways that Aβ can impact cognition while not exhibiting a linear correlation with cognitive impairment.

Furthermore, the solubility of Aβ, the composition and quantity of it in specific, disease-related forms can determine the degree of cell death and may better correlate with the clinical stage [7], [60]. Studies have marked soluble oligomeric Aβ as being a particularly noxious form [61], [62]. These can exist both extracellularly and intracellularly and can harm neurons by causing pore formation, thereby disrupting cellular calcium balance and leading to the loss of membrane potential, and can encourage apoptosis [63]. Soluble Aβ oligomers have been demonstrated to adopt a nonstandard secondary structure termed α-sheet, which forms early in aggregation and strongly correlates with toxicity [62].

The location and emergence of amyloid deposits is important to the presentation of cognitive function; some regions are more susceptible to Aβ than others. Human functional studies have suggested that the default-mode network could be one of the earliest networks affected by amyloid

burden and alterations in its activity were demonstrated in asymptomatic individuals with high levels of Aβ. This indicates that this network is particularly vulnerable to early Aβ deposition in addition to being among the first to exhibit aberrant activity [64], [65]. However, by using whole-brain immunolabeling in 5xFAD mice, Gail Canter et al. (2019) recently identified that while Aβ aggregates were detected in the default-mode network at 4 months of age, the mammillary bodies, septum and subiculum exhibited Aβ accumulation even earlier at 2 months of age, with some Aβ deposits being detected in the mammillary bodies at 1 month [66]. They showed that in this mouse model, which is based on FAD mutations, the subcortical memory structures show initial vulnerability to Aβ and that aggregates develop in progressively complex networks with age [66]. The mammillary bodies, septum and subiculum are regions that connect the hippocampus to the rest of the Papez memory circuit, which is known to be impaired in early stages of AD [67].

1.1.3 Tau Pathology

NFTs represent another of the main pathologies characteristic of AD described by Alois Alzheimer in 1907 [5]. These were discovered to be comprised primarily of the microtubule associated protein tau (MAPT) [68]–[71] which is misfolded and abnormally hyperphosphorylated [72]–[74]. Although many mutations can exist in the MAPT gene that encodes tau, these are associated with other neurodegenerative diseases such as frontotemporal dementia and other tauopathies, but not AD [75], [76]. There is no tau mutation that has been identified to cause AD, indicating that tau does not directly cause AD and emphasises the fundamental role of Aβ in the pathologic mechanisms underlying AD.

Alternative splicing of exons, 2, 3, and 10 of the MAPT gene, located on chromosome 17, generates the six recognised isoforms of tau. These vary by the number of inserts in the N-terminal region and by the number of C-terminal repeats (3R or 4R) [77]. In tauopathies, an imbalance in the amount of 3R-tau and 4R-tau exist as opposed to the equal amounts seen in healthy individuals [78], [79]. In the AD brain, equal amounts of 3R-tau and 4R-tau constitute the NFTs [79]. The

aberrant activity of tau in AD is instead deemed to be resultant of its post-translational modifications, specifically the changes in the tau phosphorylation pattern [80]. This phosphorylation at specific phospho-sites modifies the conformation of tau, promoting aggregation into an insoluble form, also known as paired helical filaments [72], [81], [82].

As discussed above, the amount of tau correlates with cognitive decline to a better degree than Aβ. The progression of NFTs through the brain is similarly less erratic. For this reason, the Braak stages which characterise the pathogenesis of AD focus on the location of NFTs. There are six stages. The first two stages (Braak I and II) describe NFTs limited to the transentorhinal region of the brain, dysfunction of which is evident in early AD [52], [83], [84]. When tau spreads to the limbic regions including the hippocampus, this is defined as stages III and IV. The last stages, V and VI, represent extensive NFTs in the neocortical regions of the brain [52].

1.1.4 Neuroinflammation

The third major contributor to AD pathogenesis is the failure of the inflammatory response to cease. Under normal conditions, it is a protective response that removes detrimental stimuli such as invasive external pathogens or misfolded proteins and aids in recovery and the restoration of homeostasis. However, in neurodegenerative diseases such as in AD, the prolonged activation of inflammation contributes to neurodegeneration [85]. In general, gliosis and neuroinflammation appear to have complicated effects that could be helpful or detrimental [86]. Many central nervous system (CNS) diseases exhibit neuroinflammation and reactive gliosis [87]. In AD, specifically, Aβ deposition results in the activation of glial cells such as astrocytes and microglia and the accompanying release of pro-inflammatory mediators [88]. These include cytokines, chemokines and reactive oxygen species, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α). These in turn can cause further activation of astrocytes and microglia in addition to extra Aβ production, resulting in a vicious cycle thought to exacerbate the pathological progression of AD, causing cell damage and apoptosis and furthering neuronal

dysfunction [89]. Furthermore, reactive microglia and astrocytes induce the deterioration of the blood brain barrier (BBB), which is vital for CNS function. If the BBB is compromised, peripheral blood leukocytes can enter the CNS, intensifying the existing neuroinflammation and other AD pathologies [90]–[92] .

Liddelow and colleagues demonstrated that reactive astrocytes, termed "A1" astrocytes, are induced through the secretion of interleukin 1α (IL-1α), TNF-α, and complement component 1q (C1q) by classically activated microglia. A1 astrocytes are regarded as more toxic compared to the more beneficial A2 astrocytes, mirroring the M1 and M2 phenotypes for microglia [93]. They do not retain the ability to support neuronal survival, outgrowth, synaptogenesis and phagocytosis, but instead prompt the death of neurons and oligodendrocytes. This occurs in AD and other neurodegenerative disorders including Parkinson's and Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis [93]. Considerable research has also linked neuroinflammation to the aggregation of phosphorylated tau [94]–[96].

Glia are typically regarded as responders to insult, rather than instigators of the disease processes. However, a more direct influence of inflammatory factors in the initial phases of pathogenesis has been proposed, implicating the role of glia in synaptic dysfunction. Disruptions in neuron-glia signalling may impact synaptic health leading to the loss of normal neuronal function and subsequent deficits in cognition [87], [97]. Furthermore, the stimulation of the inflammatory response by infectious diseases, particularly chronic or accumulative infections, have been linked to AD aetiopathogenesis. High levels of neuroinflammation can create a toxic environment leading to pathological changes in the brain [92], [98], [99]. Age, one of the major risk factors for AD, increases the body's susceptibility to infection and may help transform the normal acute response into a chronic one [100].

As discussed above, the sporadic form of AD is not driven by the three causative genes that affect the cleavage of APP as is the case in FAD [101]. In this form, the cause of the disease is far more

elusive. However, there is still a significant genetic involvement. A number of genes involved in the immune response have been connected to AD including CR1, SPI1, the MS4As, TREM2, ABCA7, CD33, and INPP5D [102]–[104]. In microglia, TREM2 (triggering receptor expressed on myeloid cells 2) regulates two branches of signalling which have influence on the reactive phenotype. One is associated with the promotion of the seemingly protective activation state of microglia and modulates phagocytosis; TREM2 has been reported to promote phagocytosis of Aβ by microglia [103, p. 2], [105]. The other moderates the inflammatory reactivity, suppressing the production and release of cytokines [103]. Variants of TREM2 can heighten AD risk by 2-4-fold, heavily implicating the role of microglia in AD pathogenesis [106]. The effect of TREM2 on tau pathology continues to be debated, with studies reporting contrasting findings regarding whether tau pathology is aggravated or alleviated by TREM2 [107], [108].

1.1.5 Apolipoprotein E

In 1993, it was discovered that polymorphic alleles of apolipoprotein E (APOE) represent a major genetic determinant of developing AD [109], [110]. ApoE is a 299-amino acid glycoprotein which regulates lipid homeostasis by transporting cholesterol and lipids between cells through the bloodstream [111]. It is expressed primarily by the liver and macrophages in the periphery and by astrocytes and other glial cells in the CNS [112]–[115]. ApoE has been established as a ligand of TREM2, binding with high affinity and thus connecting two major genetic risk factors of AD [116], [117]. The three main variants of the gene encoding the apoE protein are apoE2, apoE3, and apoE4. These variants differentially influence AD risk [114]; the presence of the ε4 allele increases the risk of an individual developing AD and at an earlier age compared to the more common ε3 allele, remaining to date the highest genetic risk factor for the disease [109], [118], [119]. The prevalence of the ε2, ε3 and ε4 isoforms are 8%, 77% and 15% respectively in the general population, however the prevalence of the ε4 allele in the AD population rises to ~40% [119]. This is because the presence of one ε4 allele increases the risk of developing AD by three to four-fold compared to ε3 homozygotes. That risk is magnified in individuals with two ε4 alleles,

putting them at an 8-fold greater risk and indicating a potential dose response of the apoE4 isoform [109], [118]. Conversely, apoE2 is regarded as protective against AD pathology [114], [119], [120]. A recent large scale study found that individuals with the APOE2/2 genotype had a 66%, 87% and 99.6% lower AD odds ratio than those with the APOE2/3, APOE3/3 and APOE4/4 genotypes respectively [121].

The detrimental effect of the apoE4 isoform in the progression of AD is well-defined, however the underlying mechanism is yet to be fully established. It has been determined that apoE isoforms vary in their regulation of Aβ clearance to a greater degree than Aβ synthesis with apoE4 being associated with a lower rate of clearance from the brain [122]–[126]. Indeed, as mentioned above, in the typically later onset, sporadic forms of AD, the build-up of Aβ is considered to be more of a consequence of defects in the clearance of amyloid from the brain, either by cellular uptake or transport across the BBB, rather than aberrant Aβ production [127], [128]. Investigation into the BBB clearance of Aβ after intracranial administration of human Aβ-42 to wild type (WT), apoE knockout (KO), and apoE3 and apoE4 targeted replacement (apoE-TR) mice revealed that the presence of apoE3 resulted in a greater clearance of Aβ from the brain; the amount of Aβ appearing in the plasma following intracranial administration was 5-fold greater than WT or apoE KO mice. A 2-fold increase in plasma Aβ levels was also seen in apoE4-TR mice compared to WT or apoE KO mice [122]. ApoE has been discovered immunohistochemically in amyloid plaques and NFTs [129], [130] and studies have indicated that apoE4 is associated with a higher neuritic plaque density [131]–[133]. Furthermore, APP transgenic mice that express human apoE isoforms display an isoform-specific effect on both the degree of Aβ build-up and deposition (apoE4 > apoE3 > apoE2) [134].

To explain how apoE isoforms differentially affect the amount of Aβ in the brain and consequently influence the risk of developing AD, several studies have been conducted. ApoE has been demonstrated to bind to Aβ, with apoE4 binding with a higher affinity than apoE3 [135] and apoE,

in particular apoE4, was reported to promote the formation of Aβ fibrils [136]. Other research provides an alternative explanation. Verghese et al. (2013) reported that apoE and soluble Aβ show little interaction in the extracellular environment and instead compete for low-density lipoprotein receptor-related protein 1 (LRP1)-dependent uptake in astrocytes. They propose that this is how apoE can differentially affect the metabolism of soluble Aβ [137].

Independently of amyloid, apoE has also been demonstrated to influence tau pathology, neuroinflammation and tau-mediated neurodegeneration *in vivo* in an isoform specific manner, with apoE4 being associated with worse pathology [138]. Homozygotes for APOE2 are at a lower risk for AD, as having two copies of the APOE2 isoform has been reported to be protective against AD, particularly against tau pathology. Far fewer tau tangles are observed in APOE2/2 carriers compared to the other APOE genotypes [121]. The magnitude of APOE's influence on tau pathology can be surmised from the recent study investigating an extremely rare mutation on the APOE3 allele, termed the Christchurch mutation (APOE3ch), which conferred protection against the autosomal dominant PS1 mutation, E280A [139]. In this paper, the authors describe the case of a Colombian woman who inherited the E280A mutation, which normally causes neurodegeneration and cognitive decline in an individual by their 40's, as a result of aberrant Aβ production. However, this woman also inherited two copies of the Christchurch mutation, and it is thought to be because of this that she is currently in her 70's experiencing short-term memory loss but remaining independent. The researchers credited this to the mutation's ability to disrupt the binding of lipoprotein receptors and other proteoglycans because of the location of the R136S mutation in the APOE gene. It was discovered that the mutation causes apoE3ch to behave in a similar manner to apoE2. Low-density lipoprotein receptor (LDLR) binding was reduced by apoE2 and apoE3ch by 98% and 60% respectively, compared to apoE3. Furthermore, both apoE2 and apoE3ch bind poorly to heparan sulphate proteoglycans which have been implicated in the promotion of Aβ aggregation and the uptake of extracellular tau [139], [140]. They found that apoE3ch demonstrated the lowest heparin binding ability relative to the other apoE isoforms,

while apoE4 displayed the highest [139], [141]. In the case study, PET imaging revealed that the woman showed very little tau pathology in her brain yet displayed a massive build-up of amyloid plaques [139].

1.2 Blood brain barrier dysfunction

1.2.1 Cerebrovasculature and blood brain barrier deterioration

Aβ can also be cleared from the brain to the systemic circulation through the BBB. Increasing evidence implicates BBB dysfunction in the pathogenesis of AD, due to altered Aβ clearance across the BBB [142], [143]. BBB damage is greater in individuals with the APOE4 isoform, contributing to the development of AD pathology [144]–[146]. APOE4 carriers exhibit augmented degeneration of pericytes compared to APOE3 carriers and non-demented controls. This correlates with the degree of loss of BBB integrity [144]. In general, cerebrovascular abnormalities are greater in APOE4 carriers resulting in further impairment of BBB function [124]. APOE4 has been implicated in hyperlipidaemia and hypercholesterolemia, which are cerebrovascular risk factors for AD [114], [147], [148]. Vascular disease has been implicated as a co-factor in AD; a considerable amount of AD cases exhibit cerebrovascular pathology, which encompasses components of the BBB [149]. Cerebral amyloid angiopathy, periventricular white matter lesions and microvascular degeneration affecting the cerebral endothelium are widespread vascular abnormalities that affect most instances of AD [150]. AD overlaps neuropathologically with cardiovascular disease and the two diseases share several risk factors in addition to APOE such as diabetes mellitus, obesity, diet, age, hypercholesterolaemia and hypertension [151].

AD can lead to structural and function changes in cerebral blood vessels. The brain demands a well-regulated supply of blood to function properly. Therefore, any structural abnormalities that hinder the supply of oxygen and the removal of toxins from the brain could exacerbate neuronal

dysfunction [152]. Ischaemic brain damage is also known to further cognitive impairment and has been linked to an elevated production of Aβ-42 [151], [153], [154].

The brain also requires a functioning BBB to be a highly specialised barrier dividing it from the peripheral nervous system and preventing potentially hazardous substances from entering the brain. The BBB also preserves the chemical composition of the neuronal "milieu" which is necessary for routine neuronal tasks, its disruption leading to dysfunction of neurons and synapses [128]. In the early 1900's, Goldmann demonstrated the existence of a barrier separating the brain and CSF with the rest of the body by injecting trypan blue dye systemically and observing its exclusion from the brain, and vice versa [155], [156]. Davson and colleagues expanded upon these original studies with dyes by describing how the brain endothelium regulates the active transport of ions [157]. Since then, many more studies have characterised the mechanisms of the BBB. It is now known that in the brain, the endothelium of cerebral blood vessels forms the BBB. This, along with associated pericytes, basement membranes, astrocytes, and microglial cells defines the neurovascular unit. Transport across the BBB is tightly regulated; tight junctions exist between the brain endothelial cells producing a diffusion barrier which impedes the paracellular influx of most blood-borne substances into the brain but allows the diffusion of small, lipid-soluble molecules in and out of the brain [158]–[160]. Large peptides require specific receptors for their transcytosis through the brain endothelium [161], including Aβ. The principal receptors for Aβ transport out of the brain are LDLR and LRP1 (discussed below). Studies have indicated that this transport through the BBB represents a major elimination route of Aβ from the brain [123], [162]–[165].

The basement membrane underlying the vasculature is comprised of the extracellular matrix (ECM), a highly structured, multifaceted complex of proteins and polysaccharides, and contributes to the structural integrity of the BBB [166]. Several factors are associated with the preservation of the ECM's integrity and that of the tissue it supports. However, in a pathological setting the functioning of the ECM becomes impaired, disrupting the integrity of the BBB. Increasing

evidence implicates the disruption of the BBB in the pathogenesis of AD, coexisting with Aβ deposition and the formation of NFTs. These disruptions include the degradation of basement membrane proteins and tight junctions and loss of pericytes [149], [167]. Dysfunction of the cerebral microvascular endothelium or other components of the neurovascular unit can affect Aβ elimination and escalate Aβ accumulation in the brain by increasing the permeability of the BBB, causing brain hypoperfusion and inducing inflammatory responses [128], [142], [143]. This in turn may further disturb the function of the BBB, forming a vicious cycle and exacerbating the development of AD [168], [169]. Transporters that carry peptides across the BBB can be additionally affected. As mentioned above, LRP1 and LDLR are the principal receptors for Aβ transport out of the brain. As such they have received a lot of attention for their role in AD pathogenesis.

1.2.2 Lipoprotein receptors

LDLR and LRP1 belong to the LDLR family of transmembrane receptors required for the uptake and removal of lipoproteins [162]. Both receptors are responsible for the clearance of Aβ from the brain [163]. The dysregulation of LRP1 and LDLR is associated with AD pathogenesis. Impairment of LRP1 function has been linked to an increase in AD risk [170], [171]. Conditional knockout of LRP1 in the brain endothelial cells lowered the clearance of Aβ injected into the brain of WT mice, increased soluble brain Aβ and reduced plasma Aβ, resulting in worsened spatial learning and memory impairment in 5xFAD mice [161]. Selective deletion of LRP1 in forebrain neurons in the APP/PS1 mouse model of AD resulted in elevated Aβ levels and intensified plaque deposition in the cortex. The authors attributed this to diminished LRP1-mediated neuronal Aβ uptake and degradation [172]. LDLR function is likewise implicated in AD pathogenesis. Overexpression of LDLR enhanced the extracellular clearance of Aβ, increased the vesicular transport to lysosomes and the cellular degradation of Aβ, reduced Aβ deposition and diminished plaque-associated neuroinflammatory responses [173], [174], while the elimination of LDLR in

5xFAD mice has been shown to increase Aβ deposition and decrease Aβ cellular uptake [173], [175].

LRP1 and LDLR are the major apoE receptors, demonstrating distinct binding affinity to apoE [119]. When human apoE is complexed with Aβ or when Aβ exists in oligomeric form, the clearance of Aβ through the BBB is diminished relative to unbound monomeric Aβ [123], [125], [176]. Moreover, Aβ complexed with apoE4 is cleared at a slower degree compared to apoE2 and apoE3 [125] and is able to penetrate the BBB via the receptor for advanced glycation end products (RAGE) transporter more readily than Aβ bound to apoE3 [122].

Lipoprotein receptors are also present in a soluble form; the soluble receptor is produced at the cell surface through proteolytic cleavage in a process called ectodomain shedding, releasing the 515kda ectodomain into the extracellular space. The soluble receptor is still able to bind to ligands but loses the capability to internalise or transport ligands intracellularly [177]–[181]. Previously, our team found a strong inverse correlation between the shedding of these receptors in the brain and Aβ transit across the BBB [182]. Following the administration of Aβ, lipoprotein receptor shedding was increased in our *in vitro* and *in vivo* prior studies, thereby further impairing the clearance of Aβ [182]. Proteolysis of receptors depletes the population of endocytic transporters available for BBB clearance and in addition increases the concentration of soluble forms of these receptors in the extracellular space, which can bind Aβ thereby extending its half-life in the brain and intensifying its detrimental effects [183], [184]. These data suggest that Aβ clearance across the BBB is mediated at least in part by lipoprotein receptor proteolysis.

Considering the close relationship between apoE and its lipoprotein receptors, it is conceivable to imagine that the manner by which apoE affects Aβ efflux through the BBB may be due to some extent to its influence on lipoprotein receptor proteolysis. Previously, our team has shown that apoE influences the extent of ectodomain shedding in an isoform-specific manner [182]. The presence of apoE2 and apoE3 attenuated Aβ-induced receptor shedding however apoE4 was less

adept at protecting the receptors than apoE2 and apoE3, the result of which was reduced clearance of Aβ across the BBB [182].

While our prior studies observed a correlation between lipoprotein receptor shedding and Aβ removal from the brain, the factors driving lipoprotein receptor proteolysis have yet to be fully explored. The mechanism by which apoE may be modulating lipoprotein receptor shedding may be due to its influence on specific enzymes. One group of protease enzymes implicated in receptor shedding is the matrix metallopeptidases (MMPs) [185]. MMP-9, in particular, has been identified as a ligand for LRP1 [186], [187] and prior studies have suggested a role for MMP-9 in the shedding of LRP1 [181], [188].

1.2.3 Matrix metallopeptidase 9

1.2.3.1 The functions of the matrix metallopeptidase family

It has been nearly 60 years since the first member of the matrix metallopeptidase family was discovered by Gross and Lapiere [189], [190]. Since then, there has been considerable research to attempt to piece together the puzzle of their assorted roles and their individual contributions to various pathological processes. It has since been found that MMPs are a family of zinc-dependent endopeptidases involved in the degradation of structural elements of the ECM such as collagen, gelatin, and elastin. This protease activity is required for physiological tissue remodelling, such as that required for embryo implantation, angiogenesis and to preserve cardiac function and integrity [191]–[193]. However, MMPs also have a well-recognised role in pathophysiological tissue remodelling and have been implicated in the pathology behind many neurodegenerative disorders and other disease states [194]. Unregulated expression of MMPs can lead to tissue injury and inflammation [195]. Currently, there are 10 different MMPs found in the mammalian CNS which

are classified into four main subgroups based on function and domain structure. These are collagenases, gelatinases, stromelysins, and film sort (MT)-MMPs [192].

1.2.3.2 MMP-9 in health and disease

MMP-9, also referred to as gelatinase B, is a member of the gelatinases group so-named due to its high specific activity for gelatins over other components of the ECM. One of its major physiological functions is to degrade proteins in the basal lamina of blood vessels in order to promote angiogenesis and is accordingly upregulated during development and wound healing [196]–[198]. However, it has received a lot of attention for its role in disease. Typically only present at low levels, it becomes substantially upregulated during many inflammatory pathologies including AD, arthritis, diabetes and cancer in which it contributes to the stimulation of the immune response and has been shown to be highly destructive [199], [200]. Indeed, there were a number of non-specific MMP inhibitors tested clinically for potential cancer treatments including BAY 12-9566 (tanomastat), a small molecular MMP-2, MMP-3 and MMP-9 inhibitor and doxycycline, a U.S. Food and Drug Administration (FDA) approved broad-spectrum MMP inhibitor currently used to treat infections such as periodontitis [201], [202].

The proteolytical activity of MMP-9 may further tissue damage following acute brain injury such as traumatic brain injury (TBI) and ischemia. MMP-9 levels are elevated after ischemia in WT mice, predominantly in vascular endothelium [203]. The degradation of components of the ECM by MMP-9 resulted in neuronal apoptosis in a transient focal cerebral ischemia model in mice [204] and MMP-9 knockout (MMP9KO) mice have been shown to be protected against both TBI and ischemia and exhibit a reduced BBB disruption [205], [206]. Research shows that MMP-9 deletion or inhibition offers favourable outcomes in multiple animal models of cardiovascular disease [193], which is a risk factor associated with the development of dementia [207].

1.2.3.3 MMP-9 in Alzheimer's disease

Overall, studies illustrate that AD patients exhibit elevated MMP-9 levels compared to control subjects. Circulating MMP-9 levels in the plasma of AD patients were found to be increased compared to controls however, levels did not differ between APOE genotype [208]. MMP-9 levels have been found to be elevated in the hippocampus and the cerebral cortex in individuals with AD [209], [210]. In the CSF of individuals with AD, MMP-9 levels were found to be higher compared with CSF from cognitively healthy elderly individuals [211]. In addition, levels of tissue inhibitor of metallopeptidases 1 (TIMP-1), a glycoprotein and natural inhibitor of MMP-9, were correspondingly lower in AD patients indicating less inhibition of MMP-9. The cognitively healthy controls carrying the APOE4 allele had elevated CSF MMP-9 levels compared to those without APOE4 [211]. As discussed above, helpful validated CSF biomarkers for AD are low Aβ-42 levels, high total tau and high phosphorylated tau protein levels [9], [10]. These biomarkers are also good for predicting AD in patients with mild cognitive impairment (MCI). In the cognitively healthy controls, higher MMP-9 levels were seen in subjects with these AD biomarkers, indicating that MMP-9 might be involved in early pathogenesis of AD [99], [211].

Increased MMP-9 expression and activity in the hippocampus has been linked to Aβ-induced cognitive dysfunction [212]. Following intracerebroventricular injection of Aβ, cognitive impairment was induced and MMP-9 levels in the hippocampus were increased. This cognitive impairment was attenuated in MMP9KO mice. Furthermore Aβ-induced neurotoxicity in primary cultured cortical neurons was diminished upon treatment with an MMP-9 inhibitor as well as in cultured neurons of MMP9KO mice compared to those from WT mice [212]. This suggests that the increase in MMP-9 expression in the hippocampus may be implicated in the development of Aβ-induced cognitive impairment.

Studies into the role of MMP-9 in AD progression have revealed that MMP-9 diminishes the integrity of the BBB by the degradation of the ECM [144], [200], [213], [214]. MMP-9 is able to

bind and proteolyse lipoprotein receptors prompting ectodomain shedding in *in vitro* and *ex vivo* studies [215]–[218]. In terms of apoE, there has been a few experiments which have investigated the relationship between MMP-9 and apoE. ApoE2 and apoE3, but not apoE4 have been reported to control the levels of certain inflammatory molecules that activate MMP-9 [219], [220], and these effects were mediated through LRP1 signalling [144], [219], [221]. Confocal microscopic analysis showed that MMP-9 accumulated in brain endothelial cells as well as pericytes in AD brains, with APOE4 carriers displaying greater levels than APOE3 carriers and non-demented controls. Correspondingly, levels of LRP1 were similarly diminished [144]. MMP-9 expression was found to be increased in apoE-TR mice in a model of TBI, with apoE4-TR mice showing higher MMP-9 expression than apoE3-TR and WT mice but less than apoE KO mice. This was associated with a loss of BBB integrity and tight junction proteins [220]. Our recent work implicated MMP-9 in lipoprotein receptor shedding and that MMP-9 function was influenced by APOE genotype [222]. MMP-9 induced lipoprotein receptor shedding in a dose-dependent manner in brain endothelial cells and freshly isolated mouse cerebrovessels. Moreover, treatment with SB-3CT, an MMP-9 inhibitor, diminished Aβ-induced lipoprotein receptor shedding in brain endothelial cells and the brains of apoE4-TR mice. SB-3CT treatment also resulted in significantly greater Aβ clearance from the brain to the periphery following intracranial administration of Aβ in apoE4-TR mice [222]. These prior studies suggest apoE influences MMP-9 function and may describe the impact of apoE isoforms on lipoprotein receptor shedding and Aβ clearance across the BBB.

1.2.3.4 MMP-9 regulation

Like other MMPs, MMP-9 is precisely regulated at several stages [223]. Under normal conditions, MMP-9 is present at low levels, however it has been found to be upregulated in many disease states, prompting its intensive scrutiny [199], [200]. MMP-9 has been found to be upregulated in endothelial cells, pericytes, neurons and glia in AD [144], [224]–[227]. MMP-9 is secreted from cells as an inactive 92 kDa proenzyme (zymogen) which is activated when cleaved by extracellular

proteinases, in an important regulatory step [228], [229]. Several activators of MMP-9 exist. These include MMP-2 [230], MMP-3 [231], MMP-7 [232], MMP-10 [233], MMP-13 [234], and the serine protease trypsin [235].

1.3 Mouse models of Alzheimer's disease

Numerous mouse models of AD that recapitulate some of the pathological features of AD have been generated. Three of these mouse models were used in the present studies, which are described here.

1.3.1.1 5XFAD mice

Mutations in the genes encoding APP and the presenilins, PS1, PS2, result in a heightened production of Aβ-42 and cause FAD. 5xFAD mice are APP/PS1 double transgenic mice that co-express five FAD mutations and additively increase Aβ-42 production resulting in a mouse model of AD with accelerated plaque development and elevated levels of cerebral Aβ-42. The five mutations are the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations in APP, and the M146L and L286V mutations in the PS1 gene, PSEN1 [236]. Of the three lines originally generated, the Tg6799 line expresses the highest levels of mutant APP and is the most widely used. The mice used in the studies presented in Chapter 4 were on a congenic C57BL/6 background as opposed to the original hybrid B6SJL background and were hemizygous for the APP and PSEN1 transgenes.

Extracellular amyloid plaques can be detected in the hippocampus, cortex and thalamus of these mice at 2 months of age and thioflavin-S-positive plaques appear between 2 and 4 months of age in the frontal, parietal and entorhinal cortices and the dentate gyrus [237], [238]. Mice exhibit progressive cerebral amyloid angiopathy from around 3 months of age [237]. Amyloid pathology is more severe in female compared to male mice [236], [239]. Spatial working memory as assessed

by the cross-maze test is impaired starting from 3-6 months of age and is exacerbated with age. Anxiety decreases progressively with age from 3-6 months in this strain of mice as determined by the elevated plus maze [240].

1.3.1.2 ApoE-TR mice

Given that APOE polymorphic alleles are the main genetic determinants of AD [109], [118], [119], mice expressing the human APOE genes are a valuable mouse model to study the role of human APOE polymorphisms in AD. In apoE-TR mice, the endogenous murine APOE gene is replaced with the human APOE2, APOE3 or APOE4 genes by gene targeting. Mice are homozygous for the APOE gene and are on a C57BL/6 Background [241]–[243]. The mice retain the endogenous regulatory sequences required for apoE production and express the human apoE protein at physiological levels. ApoE4-TR mice exhibit impaired spatial learning and memory compared to apoE3-TR mice at 3 months of age. This is attributed to defects in synaptic function rather than other aspects of AD pathology such as gliosis, amyloid deposition or NFTs, which are not different between apoE3-TR and apoE4-TR mice [244]–[246].

1.3.1.3 EFAD mice

The E2FAD, E3FAD and E4FAD mouse models were created by crossing 5xFAD mice (Tg6799 line) with apoE2-TR, apoE3-TR, and apoE4-TR mice, respectively. 5xFAD mice on the original hybrid B6SJL background were used to cross with the apoE-TR mice. This strain of 5xFAD mice develop severe amyloid pathology; elevated levels of intraneuronal Aβ (1-42) are detected at approximately 6 weeks of age followed by amyloid deposition beginning at about 2 months. Amyloid plaques rapidly develop from 2 months of age and memory impairment is apparent [236]. The EFAD animals used in the current studies were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). The EFAD mouse models were generated to study the role of APOE isoforms within the context of AD pathology. The mice remain homozygous for the APOE allele and heterozygous for the 5xFAD mutations. Overall, the EFAD mice display a less severe AD

phenotype compared to the 5xFAD line; they develop plaques in the subiculum and cortex at four months of age as opposed to two months in the 5xFAD line [247]. Early changes in the levels of Aβ are detected from 2-6 months demonstrating an age-specific effect on Aβ pathology [247]–[249]. Furthermore, EFAD mice exhibit APOE-specific effects on Aβ accumulation; the development of Aβ pathology was greater in mice carrying the APOE4 isoform compared to the APOE2 or APOE3 isoforms [247]. At 4 and 6 months, E4FAD mice exhibit significantly more plaques than the E2FAD and E3FAD models. E2FAD and E3FAD have predominantly diffuse plaques contrasting with the compact plaques in E4FAD mice [247]. Amyloid pathology is greater in female mice than male mice [250]. E4FAD mice displayed decreased levels of apoE4 in the detergent-soluble fraction suggesting that less apoE4 is lipoprotein-associated relative to apoE2 and apoE3. E4FAD mice demonstrate the most age-dependent deficits in cognitive function compared to E3FAD and E2FAD mice [251]. Liu et al. (2015) tested spatial recognition memory in E2FAD, E3FAD and E4FAD mice at 2, 4 and 6 months of age using the two-trial Y-maze and the Morris Water Maze [251]. These tests demonstrated a significant age effect for the E4FAD mice from 2-4 and 2-6 months. A significant genotype effect was observed for E4FAD mice compared to E2FAD and E3FAD mice at both 4 and 6-months of age. There were no genotype effects at 2 months [251]. The EFAD mouse model is useful for exploring the mechanisms underlying the apoE-modulated symptoms of AD pathology [252].

1.4 Clinical trials for Alzheimer's disease

There has not been a new drug approved by the FDA for treatment of AD since 2003 [253]. At present, the drugs used for treatment of AD include cholinesterase inhibitors (donepezil, galantamine and rivastigmine) and an N-methyl-D-aspartate receptor antagonist (memantine), which aim to sustain neuronal communication by enhancing the chemical pathways that are disrupted following neurodegeneration [254]–[257]. However, these can merely inhibit the

symptoms of dementia and only for a limited amount of time [257], [258]. The goal is to find disease-modifying treatments that prevent, stop or reverse AD progression [259]. Owing to the compelling nature of the amyloid cascade hypothesis, many clinical trials have been conducted on the premise of reducing amyloid plaques and overall amyloid load in the hope that this will improve downstream pathologies.

As of February 2020, there were 121 anti-AD candidates being evaluated in 136 trials. While some of these compounds aim to enhance cognition (12/121) or treat neuropsychiatric and behavioural symptoms (12/121), 97 aim to be disease modifying. Of these, amyloid remains the most popular target followed by tau [260]. Due to the aberrant production of Aβ observed in FAD as a result of mutations involved in APP processing (discussed above), a major attempt of anti-AD therapies has been to reduce the production of Aβ. As discussed above, the amyloidogenic pathway encompasses BACE. Accordingly, inhibitors of this enzyme have been thoroughly investigated. Previously tested BACE inhibitors include verubecestat and lanabecestat, which were discontinued due to lack of efficacy in mild to moderate AD clinical trials. Trials with atabecestat, evaluated for its effects in preclinical AD, were also halted when raised liver enzymes were detected in some individuals [261], [262]. The last BACE inhibitors remaining in the pipeline were cancelled last year (2019), including two phase III trials (NCT02956486, NCT03036280) and one phase II trial (NCT02322021) investigating elenbecestat, previously shown to cause a 70% reduction of CSF Aβ. The cited reason for the termination was an unfavourable risk/benefit ratio determined by its data safety monitoring board [263]. Clinical trials of CNP520, also known as umibecestat, (NCT03131453, NCT02565511) were cancelled due to a worsening of cognitive function following treatment as measured during a planned interim analysis [264].

Therapeutic antibodies represent another major category Aβ reducing drugs. The aim of these is to utilise the immune response, neutralising or enhancing the clearance of Aβ [265]. In 2019, four monoclonal antibodies were tested: aducanumab, crenezumab, gantenerumab, and solanezumab.

Aducanumab specifically targets aggregated Aβ in the parenchyma and was shown to reduce amyloid deposition in treatment groups [266], [267]. Although, aducanumab was reported to slow cognitive decline in prodromal or mild AD, it was cancelled since it did not fulfil the primary endpoint criteria [261]. Nevertheless, further analysis of additional data of the EMERGE and ENGAGE trials revealed a statistically significant effect in the highest doses of aducanumab leading Biogen to submit a Biologics License Application in early 2020 [259], [268]. At the Clinical Trials on Alzheimer's Disease conference in November 2019, Biogen stated that cognitive decline was reduced by 27% in subjects receiving the highest dose of aducanumab in the ENGAGE trial. While the antibody still requires further testing along with approval from the FDA, these initial results give support to the amyloid cascade hypothesis and the idea the amyloid can be druggable [269].

Solanezumab, a humanised immunoglobulin G1 (IgG1) monoclonal antibody against the central region of Aβ, was unsuccessful in both reducing amyloid deposits in the brain and preventing cognitive decline in mild to moderate AD [270], [271]. Both gantenerumab, a human recombinant monoclonal IgG1 antibody that targets both the amino-terminal and central regions of Aβ, and crenezumab, a humanised anti-Aβ monoclonal immunoglobulin G4 (IgG4), have a higher specificity for oligomeric and fibrillary Aβ [259], [272]. While crenezumab testing was terminated following futility analysis, trials of gantenerumab are still ongoing [273], [274]. Futility analyses evaluate data before the trial is complete and predict whether continuing the trial is worthwhile [275].

BAN2401 is a humanised IgG1 monoclonal antibody developed from the mouse mAb158 antibody and is based on the 'Arctic' APP mutation (E693G). It is selective for the large soluble Aβ protofibrils typical of this mutation [276], [277]. Previous studies in mice found that mAb158 may be neuroprotective and lower Aβ protofibril induced toxicity [278]. BAN2401 was first shown to be well tolerated in a phase I trial [279] and was enrolled in an 18-month phase II trial for early

stage AD and MCI (NCT01767311) [280]. Although after 12 months the antibody showed no cognitive benefit, futility analysis determined that it would continue for the full 18 months [281]. BAN2401 was reported to lower brain Aβ by up to 93% and slow cognitive decline by 47% on the ADAS-Cog and by 30% on the ADCOMS with a dose of 10 mg/kg twice monthly [282]. The antibody is now being evaluated in an extension to the phase 2b trial and additionally in a phase 3 trial (NCT03887455) [274]. A common issue in these antibody-based trials is amyloid-related imaging abnormalities (ARIA), a side effect in which individuals present with vasogenic oedema caused by the rapid movement and clearance of amyloid deposits. It occurs mostly in APOE4 carriers and in receivers of the highest drug doses, which is the reason that a significant number of APOE4 carriers were removed from the highest dose of BAN2401. Since APOE4 carriers exhibit a faster progression of AD, this action has caused some to have reservations about the positive results from this trial [274], [283].

Nilvadipine is a dihydropyridine-type calcium channel blocker and anti-hypertensive drug found to increase cerebral blood flow in rats, mouse models of AD and humans [284]–[286]. Previously, anti-hypertensive drugs have been linked to a reduced incidence of AD [287]. Nilvadapine has been previously demonstrated to inhibit Aβ production *in vitro*, reduce Aβ deposits *in vivo* and promote elimination of Aβ across the BBB [288], [289]. Hyperphosphorylation of tau has also been shown to be diminished by nilvadipine *in vivo* [290]. The Roskamp Institute team was involved in the evaluation of nilvadipine in mild to moderate AD disease in an 18-month phase III trial [291]. The primary outcome measures did not indicate any treatment benefit, possibly due to limitations in the study such as a too low dose and the recruitment of subjects with fully established dementia or potentially non-AD individuals due to the lack of biomarker confirmation. However, the sub-group analysis showed less cognitive decline in the mild AD group compared to placebo [291]. Further exploratory analysis suggested that very mild AD subjects responded positively to nilvadipine on both the ADAS-Cog 12 and the ADCOMS and that very mild and mild AD patients treated with nilvadipine showed beneficial effects on memory and language traits, respectively

[292]. Moreover, APOE4 carriers exhibited less cognitive decline compared to non-carriers after treatment with nilvadipine [291].

Numerous trials directed at non-amyloid based mechanisms have also proved futile. These include anti-tau-based therapies, natural substances such as vitamins, fish oils or botanical compounds, hormones and anti-inflammatory drugs [293]–[295]. An inhibitor of tau aggregation was found to provide no treatment benefit in mild to moderate AD [296]. A trial investigating pioglitazone, an insulin sensitising agent, in preclinical AD was cancelled and an inhibitor of the tyrosine kinase, Fyn, showed no differences upon treatment in any clinical measure [261].

Even with the relative success of aducanumab and BAN2401, many anti amyloid therapies have failed to meet their endpoint criteria, which has led many to doubt the suitability of this approach. However, a closer evaluation points towards technical limitations as opposed to Aβ being an erroneous treatment target [253], [295], [297]. There are several reasons postulated to explain these failures, including not intervening early enough, incorrect drug doses, flawed treatment targets and not comprehending in depth the contribution of Aβ and other factors in AD [259], [296]. Indeed, many drugs were reported not to have passed biochemical effect thresholds necessary for clinical improvement [295]. It is important for drugs to be tested at the stage of the disease where they would be predicted to be most effective in addition to being assessed at a dose that has been pre-determined in early trials to have sufficient target engagement [298]. This has been recently highlighted by the exploratory analysis following treatment with nilvadipine in AD, demonstrating differential responses related to disease severity at the beginning of treatment [292].

To enhance future clinical trials, technical and methodological improvements and revised diagnostic criteria are required. For example, including prodromal or pre-symptomatic individuals at high risk of developing the disease and using better biomarkers will help in developing preventative treatments [299]. Both objective subtle cognitive difficulties and tau-PET have been reported to be predictive of future AD pathology such as Aβ accumulation, neurodegeneration

and atrophy [300], [301]. In humans, the pre-symptomatic phase of AD can last for many years, until it is thought that the accumulation of Aβ reaches the point where it elicits the activation of neurotoxic cascades which ultimately lead to cell death and dementia [302]. High levels of cortical Aβ are detected in cognitively healthy individuals as well as those with dementia, signifying that extensive Aβ deposition, at least in the cortex, can precede cognitive impairment [303]. Early stage pathology including neuroinflammation and tau pathology can also occur before the manifestation of clinically defined dementia [302]. Once clinical symptoms arise, Aβ has accumulated over many years and extensive neurodegeneration has already occurred, potentially rendering the treatment ineffective [298]. This suggests that early intervention may be necessary for AD treatment. Furthermore, due to the heterogenous nature of AD, clinical trial simulators or longer duration of treatment, although costly, may be necessary to overcome variability in measurements and detect efficacy from drug candidates [297].

With the lack of success that was apparent in many of the AD trials, there has been a rising appreciation of just how complex and diverse the pathology is. There is a dynamic interplay between the different components and signalling pathways of the disorder which seem to vary a great deal between individuals [304], [305]. Accordingly, it may be necessary to focus on more than one target, opting for combination therapies for the treatment of AD [306], [307]. The elimination of Aβ, tau, neuroinflammation or any other AD pathology alone after homeostasis has already been severely disrupted may not be sufficient to halt or reverse cognitive decline. Future treatments may need to also act downstream of the main pathologies, working to restore normal brain function. Hence it is vital to understand AD pathology in depth, filling in the necessary gaps about the mechanisms involved in order to assist the development of potential therapies [308].

1.5 Aims of the studies

APOE is a significant risk factor for the development of AD, however the underlying mechanisms are not well defined [109], [110]. Prior work by our group suggested that apoE may be regulating MMP-9 disposition [222]. Since MMP-9 can be regulated at multiple levels [223], this thesis examined the effect of apoE isoforms on different aspects of MMP-9 regulation with the hypothesis that apoE4 is less efficient than other apoE isoforms in modulating MMP-9 function. In addition, since MMP-9 has been previously implicated in AD pathology [208], the targeting of MMP-9 in mice with an AD phenotype is anticipated to have a beneficial effect on cognitive function.

This thesis can be divided into 2 major parts:

Part 1: To delineate the molecular mechanisms by which the apoE isoforms can differentially modulate MMP-9 function using *in vitro* and *ex vivo* methods.

As discussed above, MMP-9 is precisely regulated at several levels including induction and secretion of a proenzyme, activation of the proenzyme by proteolytic cleavage of the pro domain and modulation of active MMP-9 [223]. The purpose of Chapters 2 and 3 was to examine the relationship between apoE and MMP-9 and further our understanding of the role of apoE in AD pathophysiology. The proposed mechanisms by which apoE could be regulating MMP-9 disposition which were investigated in Chapters 2 and 3 are summarised in Figure 1.3.

Part 2: To further elucidate the role of MMP-9 in the pathology of AD and to investigate MMP-9 as a possible target for the treatment of AD.

The aims of Chapter 4 were to inhibit MMP-9 *in vivo* and investigate the effects on behaviour, in particular cognitive function, Aβ-40 and Aβ-42 levels in the brain and plasma and LRP1 and LRP2 levels in the brain.

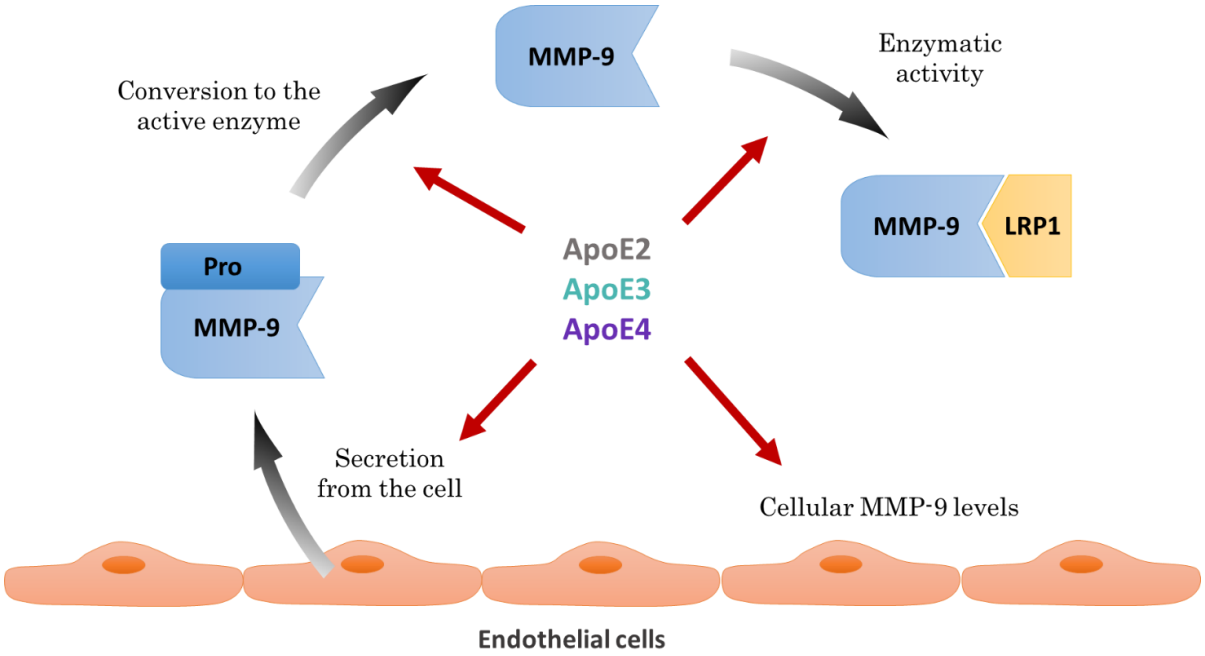


Figure 1.3: Schematic diagram depicting the proposed regulatory mechanisms of MMP-9 by which apoE could be influencing MMP-9 disposition.
MMP-9 can be regulated at multiple levels including the modulation of MMP-9 levels, secretion of the proenzyme from the cell, conversion of the proenzyme to the active form and enzymatic activity. Since MMP-9 exerts its detrimental effects at the blood-brain-barrier, endothelial cells were focused on as a source of MMP-9 production and release.

Chapter 2: Effect of apoE on the expression and secretion of MMP-9 in human and mouse brain tissue

2.1 Introduction

As discussed in Chapter 1, elevated MMP-9 levels have been reported in numerous inflammatory disease states including AD [200], [208], [309]. In AD, patients have been reported to exhibit elevated MMP-9 expression and activity in the plasma, hippocampus and frontal cortex compared to control subjects [208]–[210], [309], [310]. Moreover, Aβ has been shown to directly induce the expression and gelatinolytic activity of MMP-9 [310], [311]. Increased MMP-9 levels in AD have also been attributed to the heightened levels of proinflammatory cytokines and oxidative damage present in AD [222], [224], [312]–[315].

Our group and others have shown that apoE isoforms vary in their ability to remove Aβ from the brain with apoE4 being associated with reduced BBB clearance from the brain [122]–[126]. Our team also found an isoform-specific effect of apoE on lipoprotein receptor shedding (LRP1 and LDLR) with a rank order of apoE4>apoE3>apoE2 [182]. Collectively, these findings showed an inverse relationship between lipoprotein receptor shedding in the brain and Aβ elimination across the BBB, one that is APOE-genotype specific [182]. Our previous studies suggest that the effect of apoE on these processes may be mediated through MMP-9 [222]. MMP-9 is able to bind and proteolyse lipoprotein receptors prompting ectodomain shedding based on *in vitro* and *ex vivo* studies [215]–[218]. The proteolytic shedding of LRP1 and LDLR by MMP-9 impairs their ability to transport Aβ out of the brain [182], [222]. For this reason, coupled with the known secretion of MMP-9 from endothelial cells [316]–[318], the investigation of MMP-9 expression was focused to the cerebrovasculature. Parts of the data and conclusions presented in this Chapter have been peer-reviewed and published [319].

While our previous studies indicated that apoE differentially regulates MMP-9 disposition [222], the mechanism by which this occurs is not yet known. Considering the above data, one potential way in which apoE may influence MMP-9 disposition could be the modulation of MMP-9 expression levels. Studies indicate that there are considerable differences in gene expression patterns associated with different APOE isoforms which contribute to the risk conferred by each genotype [320], [321]. It has previously been demonstrated by confocal microscopic analysis that MMP-9 accumulated in brain endothelial cells as well as pericytes in human AD compared to control subjects. Within the AD samples, APOE4 carriers displayed greater levels of MMP-9 in these cerebrovascular cells than APOE3 carriers [144]. Furthermore, brain MMP-9 levels have been reported to be increased in apoE4-TR mice relative to apoE3-TR and apoE2-TR mice [219]. To expand upon these studies, in this Chapter, MMP-9 levels in AD across the APOE genotypes, both heterozygous and homozygous (APOE2/2, APOE2/3, APOE3/3, APOE3/4, APOE4/4) were investigated. The effect of APOE genotype on MMP-9 levels in non-demented controls was also examined to assess any APOE effects in the absence of AD pathology.

In addition to increasing the expression levels of MMP-9, Aβ has been shown to be a potent stimulator of MMP-9 secretion from endothelial cells, neurons and glial cells [224]–[227]. Since the primary function of MMP-9 is to degrade proteins in the basal lamina of blood vessels in order to promote angiogenesis [196]–[198], [318], it follows that there would be a high production and secretion of the protease in endothelial cells and the surrounding cerebrovasculature [318]. In the current studies phorbol-12-myristate-13-acetate (PMA) was used to stimulate the secretion of MMP-9 from cells since it is a known stimulator of MMP-9 secretion [316], [317], [322]. PMA is a phorbol ester which induces MMP-9 expression via a protein kinase C-α (PKC-α)-dependent signalling cascade, thereby promoting angiogenesis [323]–[325].

TNF-α [326], [327], transforming growth factor β (TGF-β) [326], [328], IL-1α [316], IL-1β [329] and angiogenic factors including fibroblast growth factor-2 and vascular endothelial growth factor

[318] have also been reported to induce MMP-9 secretion from cells. The induction of MMP-9 can be attenuated; in some cell types forskolin has been reported to suppress MMP-9 induction [316]. MMP-9 is typically secreted from the cytosol as a proenzyme which is activated outside the cell. Following stimulation of human cultured mast cells with PMA, proMMP-9 was detected at the earliest measured time point of 12 hours post stimulation, increasing up to 96 hours, however active MMP-9 was only detected 24 hours after stimulation [322]. However, both pro and active MMP-9 have also been detected within vesicles shed from human umbilical vein endothelial cells, being released into the extracellular environment as early as 4 hours following stimulation by angiogenic factors [318].

The cellular regulation of MMP-9 is complex and strictly controlled, and MMP-9 levels can be regulated through the induction of the pro-enzyme, either through increasing its expression or by altering its secretion from the cell. Since these processes can be enhanced by certain cellular factors, as discussed above, and given the evidence of an effect of APOE genotype on MMP-9 disposition, it was investigated in this Chapter whether apoE can influence MMP-9 expression and secretion in an isoform specific manner.

2.2 Materials and methods

2.2.1 Materials

Enzyme linked immunosorbent assay (ELISA) kits for mouse MMP-9 were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). Human Aβ(1-42) and ELISA kits for human MMP-9 were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Human MMP-9 activity assays were purchased from QuickZyme Biosciences (Leiden, The Netherlands). Dextran was purchased from MilliporeSigma (St. Louis, MO, USA). Halt enzyme inhibitor cocktails, the bicinchoninic acid (BCA) protein assay and Hanks Balanced Salt Solution (HBSS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2.2 Human cortex samples

Human cortex samples (inferior frontal gyrus) were obtained from the autopsied brains of AD and non-demented subjects with different APOE genotypes as summarised in Table 1. The de-identified human brain specimens were provided by the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona and the University of Maryland Brain and Tissue Bank which is a Brain and Tissue Repository of the NIH NeuroBioBank (Baltimore, MD) and the Mount Sinai NIH Brain and Tissue Repository (New York, NY).

APOE	**Non-Demented**		**Alzheimer's Disease**			
	Mean age ± StDev	**N (M/F)**	**Mean age ± StDev**	**N (M/F)**	**Age of Onset**	**Amyloid burden**
2/2	79.0 ± 19.49	10(3/7)	82.67 ± 21.22	3(2/1)	N/A	N/A
2/3	79.8 ± 10.84	10(4/6)	85.5 ± 10.05	10(3/7)	78.5 ± 3.45	11.4 ± 1.28
3/3	79.8 ± 15.53	10(9/1)	84.5 ± 5.83	16(5/11)	76.9 ± 2.84	14.2 ± 0.25
3/4	82.0 ± 9.02	10(7/3)	79.4 ± 10.18	17(10/7)	72.8 ± 3.14	12.9 ± 0.33
4/4	51.6 ± 16.17	10(5/5)	80.8 ± 7.80	12(6/6)	72.9 ± 3.31	13.5 ± 0.51

Abbreviations: APOE, apolipoprotein E genotype; StDev, Standard deviation; M, male; F, female.

Table 1. Demographic details of human brain cortex samples.

The tissue was collected by various banks in accordance with the approved IRBs (Institutional Review Boards), and was received from the banks under the conditions below:

The studies in this thesis did not constitute research involving human subjects as the studies did not involve intervention or interaction with living individuals or identifiable private information. The current studies utilised frozen human brain specimens acquired from various tissue banks. The investigator did not intervene nor interact with the subjects in obtaining the de-identified specimens. The specimens were not linked to any protected health information and no identifiable private information was collected.

University of Maryland Brain and Tissue Bank
The University of Maryland Institutional Review Board
IRB Protocol Number: 00042077

Banner Sun Health Research Institute
Western Institutional Review Board
IRB Protocol Number: 20120821

More details regarding the origins of the human samples are listed in the acknowledgements.

2.2.3 Animals

ApoE-TR mice were purchased from Taconic Biosciences (Rensselaer, NY) and allowed to adapt to the vivarium for 2 weeks prior to any experimental procedures. The apoE-TR mice were created by targeted replacement of the endogenous murine APOE gene with human APOE2, APOE3 or APOE4 [330]. These mice retain the endogenous regulatory sequences required for apoE production and express the human apoE protein at physiological levels. The EFAD animals were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). To generate the EFAD mouse model, transgenic mice expressing 5 familial AD mutations (5xFAD, Tg6799 line) were crossed with apoE4, apoE3, and apoE2-TR mice, producing the E4FAD, E3FAD, and E2FAD mouse models respectively, as previously described [247]. Mice were housed under standard laboratory conditions (23±1°C, 50±5% humidity, and a 12-hour light/dark cycle) with free access to food

and water throughout the study. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

2.2.4 Isolation of brain fractions

Frozen human cortex samples and mouse brains were homogenised and the cerebrovasculature was isolated using a step-wise density gradient extraction process as previously described [182]. Briefly, human or mouse brain samples were homogenised in cold HBSS using a Dounce homogeniser. The homogenates were suspended in HBSS with 20% dextran and centrifuged for 15 minutes at 6000g and 4 °C. The cerebrovascular pellet at the bottom of the tube was gently rinsed in HBSS and either collected with lysis buffer (mammalian protein extraction (M-PER) reagent + 1% ethylenediaminetetraacetic acid (EDTA) + 0.2% phenylmethylsulphonyl fluoride (PMSF) (Thermo Scientific, USA)) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA) and stored at -80 °C prior to analysis, or immediately resuspended in endothelial cell medium and used for the *ex vivo* studies described below.

2.2.5 Measurement of active and total MMP-9 levels in mouse and human cerebrovasculature

Total MMP-9 levels in the cerebrovasculature and the homogenate from mouse and human brain samples were detected using an MMP-9 ELISA. Absorbance was detected using a BioTek Powerwave XS microplate apparatus at a wavelength of 450nm. A quantitative substrate-based MMP-9 activity assay (QuickZyme Biosciences) was used to measure active MMP-9 levels in the cerebrovasculature from human brain samples. This assay provides a measure of MMP-9 activity in a biological sample by capturing MMP-9 present in the sample using an antibody followed by the addition of a proenzyme as a substrate, which upon activation releases colour from a chromogenic peptide substrate. Absorbance was then detected using a BioTek Powerwave XS microplate apparatus at a wavelength of 405nm. MMP-9 levels were normalised to total protein content, as determined by the BCA assay.

2.2.6 AB-42 injected apoE-TR mice

Using a standard process to limit aggregation, as previously described [222], [331], lyophilised Aβ peptides were solubilised in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain a monomeric/dimeric sample and reduce the development of β-sheet structures. Briefly, 1 mg of each lyophilised peptide was dissolved in 1 ml of ice-cold HFIP. The peptides were left to air dry for 1 hour in a chemical fume hood followed by further drying in a speed-vac centrifuge for 30 min. The resulting clear film was resuspended in 100% dimethyl sulfoxide (DMSO) to a concentration of 1 mM and stored at -80 °C. ApoE2, apoE3 and apoE4-TR mice were stereotaxically injected with 3 μl of monomeric 1 mM human Aβ(1–42) or 100% DMSO bilaterally into the caudate putamen of the brain (0.5 mm anterior to the bregma, 2 mm lateral to the midline, and 3 mm below the surface of the skull). Ten minutes after intracerebral administration of human Aβ(1–42), mice were euthanised and the brain (excluding the cerebellum) was extracted and immediately snap frozen in liquid nitrogen. To assess MMP-9 levels in the cerebrovasculature, mouse brains were processed to isolate various brain fractions, as described above.

2.2.7 Zymographic analysis of EFAD spleen samples

Lysis buffer (M-PER + 1% EDTA + 0.2% PMSF (Thermo Scientific, USA)) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA) was added to spleen samples collected from EFAD mice before they were homogenised via sonication (Sonic Dismembrator model 100, T). Samples were centrifuged to remove cell debris before analysis by gelatin zymography to determine pro and active MMP-9 levels. Equal protein quantities of each sample (50 μg) were incubated with Gelatin-Sepharose® 4B (GE Healthcare, Chicago IL) to concentrate MMP-9. Samples were incubated with the beads for 1-2 hr at room temperature with rotation, and then centrifuged at 6000 rpm for 2 min. Gelatinases were eluted in equal amounts (25 μL) of 1X Zymogram sample buffer (Bio-Rad, Hercules, CA, USA) before being separated on a 10% precast polyacrylamide gel with gelatin (Thermo Fisher Scientific, Waltham, MA, USA). The gel was incubated in Triton X-100 (Zymogram Renaturation Buffer, Thermo Fisher Scientific,

Waltham, MA, USA) for 30 minutes at room temperature with gentle agitation to renature the proteins. The gel was next incubated in development buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl2, 0.02% Brij-35 (Zymogram Development Buffer, Thermo Fisher Scientific, Waltham, MA, USA) for 18h at 37 °C to initiate enzyme activity. The gel was stained with 0.5% Coomassie blue (Bio-Rad, Hercules, CA, USA) for one hour and washed in destaining solution (45% deionised water, 45% methanol, 10% acetic acid) before being scanned with the Universal Hood II (Bio-Rad, Hercules, CA, USA). Bands of MMP-9 activity were visualised as clear lysis zones on the gel which were quantified by densitometry using Image Lab 6.1.

2.2.8 Collection and enrichment of human lipidated apoE

The mixed glial cultures were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). Cortical glial cultures were prepared from apoE2-TR, apoE3-TR, or apoE4-TR mice as previously described [332]. Briefly, mixed glial cells from 1–2-day-old neonatal apoE-TR pups were plated in 150cm^2 flasks (~1½ brains per flask) in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium containing 10% foetal bovine serum (FBS), L-glutamine (2mM) and 1% penicillin/streptomycin. Medium was changed every 3-5 days [332], [333]. Upon confluency, cultures were collected and grown in 75cm^2 flasks until confluent. Cells were washed and incubated with serum-free media for 72 hours [333]. Following the incubation, glial-conditioned media was collected and centrifuged at 1,000g for 3 min to remove any residual cells before being concentrated (10x) using the Vivaspin 15 centrifugal concentrator with a molecular weight cut-off of 10,000 Da (Sartorius Mechatronics Corp., Bohemia, NY). The resulting concentrate was analysed for apoE content using a human apoE ELISA as per the manufacturer's instructions and used to treat human brain microvascular endothelial cells (HBMECs) in the *in vitro* studies described below.

2.2.9 Effect of APOE genotype on total secreted MMP-9 and secreted active MMP-9 in vitro

HBMEC were seeded at 100,000 cells per cm^2 onto fibronectin-coated 12-well plates in endothelial cell medium containing 5% FBS, 1% endothelial cell growth supplement and 1% penicillin/streptomycin solution. When approximately 90% confluent, cells were washed twice thoroughly in phosphate-buffered saline (PBS) before being treated with serum-free endothelial cell medium containing lipidated apoE2, apoE3 or apoE4 (25 ng/ml) collected from mixed glial cultures (as described above) and incubated for 1h at 37 °C and 5% CO_2. Cells were then treated with PMA (1μM), human Aβ(1-42) (2μM) (known stimulators of MMP-9 secretion) or dimethyl sulfoxide (DMSO) (0.5%) for 18h at 37°C and 5% CO_2. After the incubation period, the conditioned extracellular media was collected, centrifuged to remove cell debris and analysed for total secreted MMP-9 using an ELISA and for endogenously active and total MMP-9 using a quantitative substrate-based MMP-9 activity assay (described above).

2.2.10 Effect of APOE genotype on total secreted MMP-9 ex vivo

Cerebrovasculature was isolated from apoE3-TR and apoE4-TR mice through a step-wise density gradient extraction process, as described above, and seeded at 2 million cells per cm^2 in 48-well plates in serum-free endothelial cell medium. After a 4-hour incubation at 37°C and 5% CO_2, the media and cell debris was removed and serum-free endothelial cell medium containing PMA (1μM), human Aβ (1-42) (2μM) or DMSO (0.5%) was added to the cells. After 24 hours at 37°C and 5% CO_2, the conditioned media and cell lysates were collected, and the conditioned media was analysed for total secreted MMP-9 using an ELISA.

2.2.11 Tissue processing

All mice were humanely euthanised, and their brains were collected and fixed in 4% paraformaldehyde (PFA) for 48 h as previously described [334]. The hemispheres were processed in a Sakura Tissue-Tek VIP (Leica Biosystems Inc., IL, USA) vacuum infiltration processor before

being embedded in paraffin with the Sakura Tissue-Tek (Leica Biosystems Inc., IL, USA) and stored at 4 °C for 2 days for subsequent cutting with a Leica RM2235 microtome (Leica Biosystems Inc., IL, USA). All brains were cut at a thickness of 12 μm. Sagittal slices were mounted on glass slides and dried for 48 h at 37 °C for immunofluorescence staining and confocal imaging.

2.2.12 Immunofluorescence

Paraffin sections were washed in two baths of histoclear (National Diagnostics, USA) and progressively rehydrated with ethanol gradients and PBS (Sigma Aldrich, MO, USA). Brain sections were subjected to antigen retrieval for 7 min in glycine buffer (pH 3) at 100 °C. Sections were then blocked in PBS containing 100% SeaBlock (EastCoast Bio, MA, USA), 0.5mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 for 1 h. Sections were incubated in PBS containing 10% SeaBlock, 0.33mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 and the respective panel of primary antibodies overnight at 4 °C. The following antibodies were used: ab38898 (anti-MMP-9, 1:200, Abcam, Cambridge, MA, USA) and AF3628 (anti-platelet endothelial cell adhesion molecule (CD31/PECAM-1), 1:1000, Novus Biologicals, Centennial, CO, USA). After three washing steps in PBS for 5 min, sections were incubated in PBS containing 10% SeaBlock, 0.33mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 and the respective panel of secondary antibodies for 1 h in the dark at room temperature in a humidified chamber. The following secondary antibodies were used: donkey anti-rabbit, anti-goat conjugated to Alexa 488 and 568, respectively (1:1000, Life technologies). After three washing steps in PBS for 5 min, sections were mounted in Fluoroshield (Sigma Aldrich, MO, USA). All images were acquired using the confocal microscope LSM 800 (Carl Zeiss AG, Germany), the ZEN Blue 2.3 (Carl Zeiss AG, Germany) software and a 20× objective. The acquisition settings were kept the same for all genotypes within the same experiment. For qualitative analysis of MMP-9 immunoreactivity, orthogonal projections of cortical brain regions from E3FAD and E4FAD mice (n = 4 for each genotype, all female mice, 6 month of age) were analysed. Six images were generated for each mouse. Averages calculated from the six images were used for statistical analysis.

2.2.13 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Data was checked for normality and statistical significance was determined by analysis of variance (ANOVA) followed by the two-stage step-up method of Benjamini, Krieger and Yekutieli (BKY) unless otherwise stated. A p-value lower than 0.05 was used to indicate a statistically significant difference. Statistical analyses were performed with GraphPad Prism 8.

2.3 Results

2.3.1 MMP-9 expression and activity across APOE genotype in human cerebrovasculature

Isolated cerebrovasculature from human brain tissue (AD and non-demented controls) showed MMP-9 expression was significantly higher (2-fold) in the cerebrovasculature from AD specimens compared to control samples (Fig. 2.1a). Upon stratification by APOE genotype, MMP-9 expression in AD subjects was markedly elevated in ε4 carriers compared to non ε4 carriers, with the APOE3/4 genotype showing significantly higher MMP-9 expression levels compared to subjects with the APOE2/3 and APOE3/3 genotypes (*p<0.05), in addition to being significantly higher than the corresponding APOE3/4 control subjects (**p<0.01) (Fig. 2.1b). There was no such APOE genotype difference observed in the control samples.

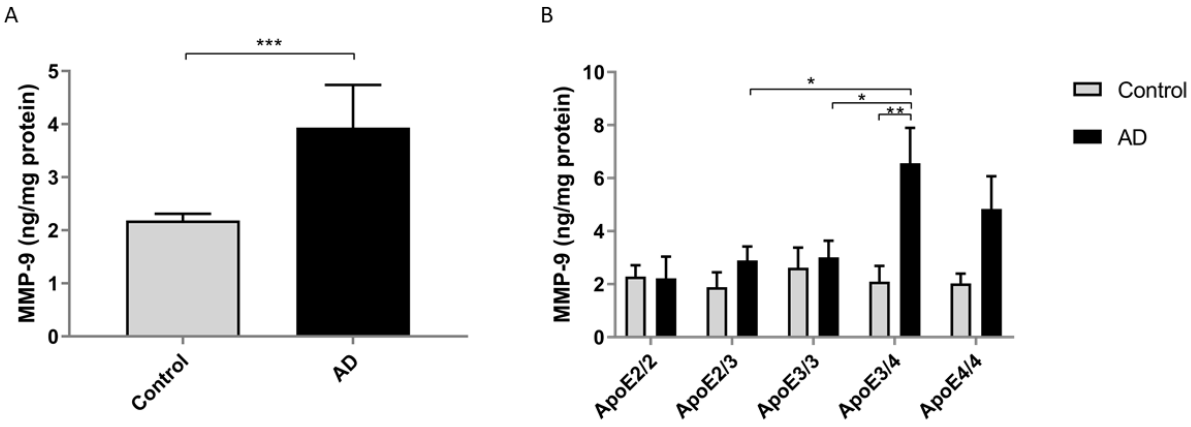


Figure 2.1: APOE genotype effect on total MMP-9 levels in the cerebrovasculature of human brain tissue in AD and control subjects.
Levels of total MMP-9 were found to be elevated in (A) Alzheimer's disease (AD) subjects compared to non-demented controls and (B) APOE4 carriers with AD. MMP-9 levels in the cerebrovasculature were determined using an ELISA. Values were normalised to total protein concentration and represent mean ± SEM. (A) n = 49 (control) n = 55 (AD). **p<0.01 as determined by an unpaired t-test. (B) Control subjects: APOE2/2: N = 10, APOE2/3: N = 9, APOE3/3: N = 10, APOE3/4: N = 10, APOE4/4: N = 10. AD subjects: APOE2/2: N = 3, APOE2/3: N = 10, APOE3/3: N = 13, APOE3/4: N = 17, APOE4/4: N = 12. *p>0.05, **p<0.01, ***p<0.001, as determined by a two-way ANOVA and the BKY procedure.

To assess MMP-9 activity in relation to APOE genotype, the human brain specimens were analysed using a quantitative substrate-based MMP-9 activity assay which measures endogenously active MMP-9 within biological samples. The levels of endogenously active MMP-9 were increased in the AD specimens compared to non-demented controls (Fig. 2.2a). In terms of APOE, AD subjects with the APOE3/4 and APOE4/4 genotypes exhibited significantly higher levels of active MMP-9 compared with their corresponding control subjects. Furthermore, ε4 carriers with AD displayed significantly elevated levels of active MMP-9 compared to non-APOE4 AD subjects. Lastly, no APOE genotype differences were observed within the control population (Fig. 2.2b).

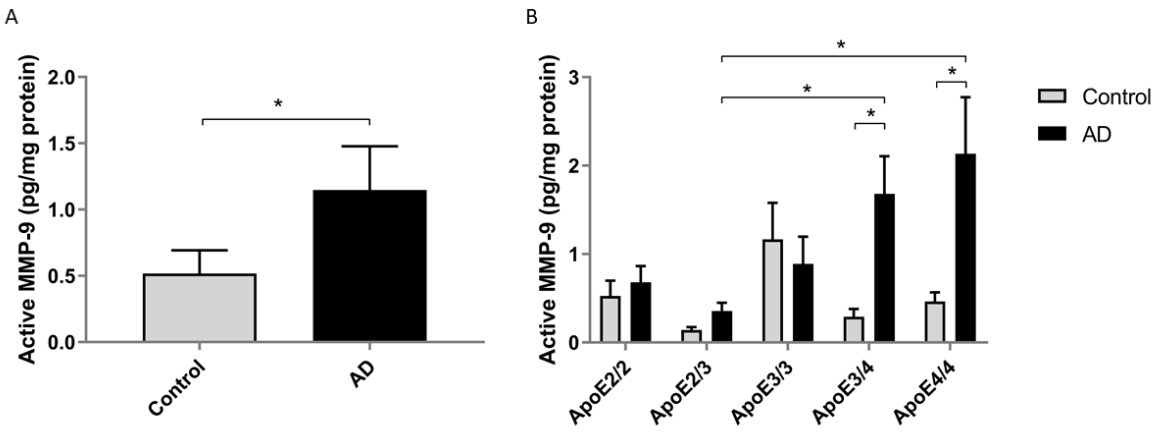


Figure 2.2: APOE genotype effect on active MMP-9 levels in the cerebrovasculature of human brain tissue in AD and control subjects.
Using a quantitative substrate-based MMP-9 activity assay, levels of endogenously active MMP-9 were found to be elevated in (A) Alzheimer's disease (AD) subjects compared to non-demented controls and (B) APOE4 carriers with AD. Values were normalised to total protein concentration and represent mean ± SEM. (A) n = 44 (control) n = 48 (AD). *$p<0.05$ as determined by an unpaired t-test. (B) Control subjects: APOE2/2: N = 8, APOE2/3: N = 8, APOE3/3: N = 13, APOE3/4: N = 8, APOE4/4: N = 7. AD subjects: APOE2/2: N = 3, APOE2/3: N = 9, APOE3/3: N = 11, APOE3/4: N = 15, APOE4/4: N = 10. *$p<0.05$ as determined by a two-way ANOVA and the BKY procedure.

2.3.2 MMP-9 expression across APOE genotype in EFAD mice

For a global view of changes in MMP-9 levels in relation to APOE genotype in an AD environment, whole brain homogenate of EFAD mice was examined via ELISA. To investigate whether MMP-9 levels are altered at different stages of AD progression, tissue from mice aged 10, 40 and 70 weeks was analysed. There were no differences in MMP-9 levels between E4FAD and E3FAD mice at any age point. While no differences in APOE genotype were detected, levels of MMP-9 were elevated in E4FAD mice at 70 weeks of age compared to 10 weeks of age (Fig. 2.3a). E2FAD mice could only be analysed at 40 weeks of age due to a lack of availability of this genotype, however again, no APOE genotype differences in MMP-9 were observed (Fig. 2.3b).

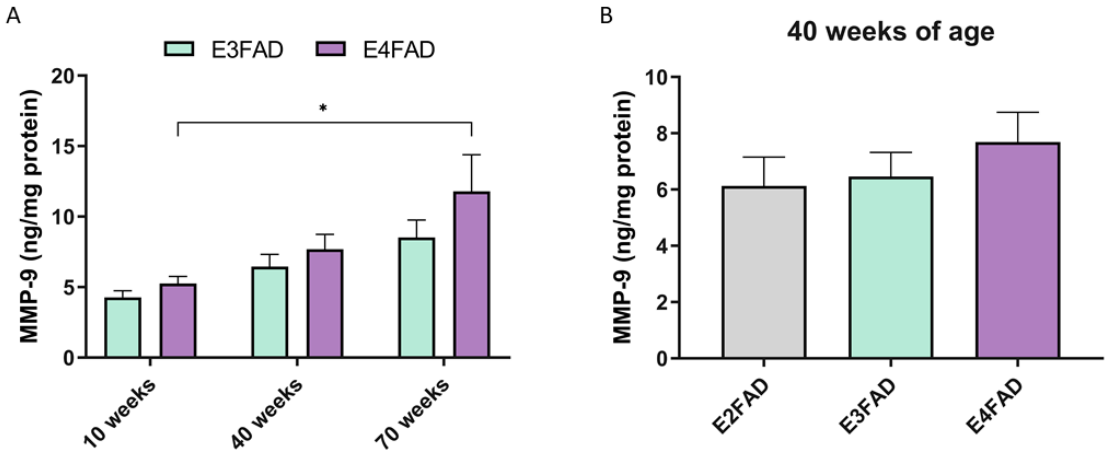


Figure 2.3: MMP-9 expression in mouse homogenate tissue from EFAD mice at 10, 40 and 70 weeks of age.
Levels of MMP-9 were measured in the whole brain homogenate of (A) E3FAD and E4FAD mice at 10, 40 and 70 weeks of age and (B) E2FAD, E3FAD and E4FAD mice at 40 weeks of age. MMP-9 levels were found to be higher in E4FAD mice at 70 weeks of age relative to 10 weeks of age. MMP-9 levels were determined using an ELISA. Values were normalised to total protein concentration and represent mean ± SEM (E2FAD: N=4, E3FAD & E4FAD: N=8 per age group). (B) *$p<0.05$ as determined by ANOVA and the BKY procedure.

Focusing this examination on cerebrovasculature tissue from EFAD mouse brains revealed that total MMP-9 levels were increased in E3FAD and E4FAD mice compared to E2FAD mice at 40 weeks of age (**p<0.01) (Fig. 2.4b). Analysis of E3FAD and E4FAD mice at 10, 40 and 70 weeks of age by 2way ANOVA indicated a significant difference between APOE genotype ($F(2, 41) = 3.474$, $p < 0.05$), however this was not significant following post hoc testing (Fig. 2.4a).

To further probe potential APOE genotype differences in E3FAD and E4FAD mouse cerebrovasculature, MMP-9 levels were investigated specifically within brain endothelia using immunofluorescence and confocal microscopy. The brain cortices of 6-month-old E3FAD and E4FAD mice were analysed and MMP-9 immunoreactivity was detected in cortical endothelial cells (indicated by white arrows) (Fig. 2.5). There was a 56% increase in MMP-9 immunoreactivity in cortical endothelial cells from E4FAD mice compared to E3FAD mice (*p<0.05) (Fig. 2.5c). Furthermore, the total area of MMP-9 in the endothelial cells appeared to be greater in E4FAD compared to E3FAD mice, although this was not significant ($p = 0.06$) (Fig. 2.5d). For a measure of the physiological baseline levels of MMP-9 activity in EFAD mice, spleen samples were analysed via zymography. No differences were detected between E3FAD and E4FAD mice (Fig. 2.6a, b).

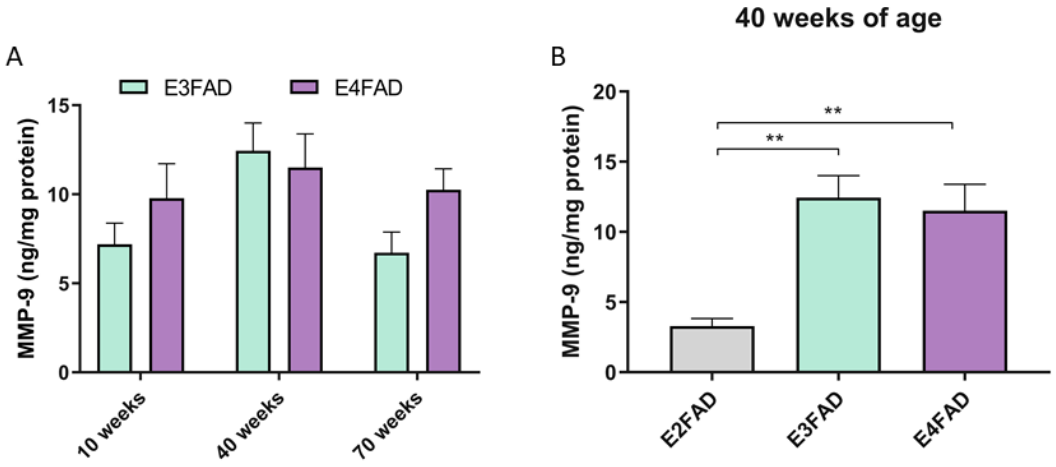


Figure 2.4: MMP-9 expression in mouse cerebrovasculature tissue from EFAD mice at 10, 40 and 70 weeks of age.
Levels of MMP-9 in the cerebrovasculature were measured in (A) E3FAD and E4FAD mice at 10, 40 and 70 weeks of age and (B) E2FAD, E3FAD and E4FAD mice at 40 weeks of age. MMP-9 levels were found to be higher in E3FAD and E4FAD mice relative to E2FAD mice at 40 weeks of age. MMP-9 levels were determined using an ELISA. Values were normalised to total protein concentration and represent mean ± SEM (E2FAD: N=4, E3FAD & E4FAD: N=8 per age group). **p<0.01 as determined by ANOVA and the BKY procedure.

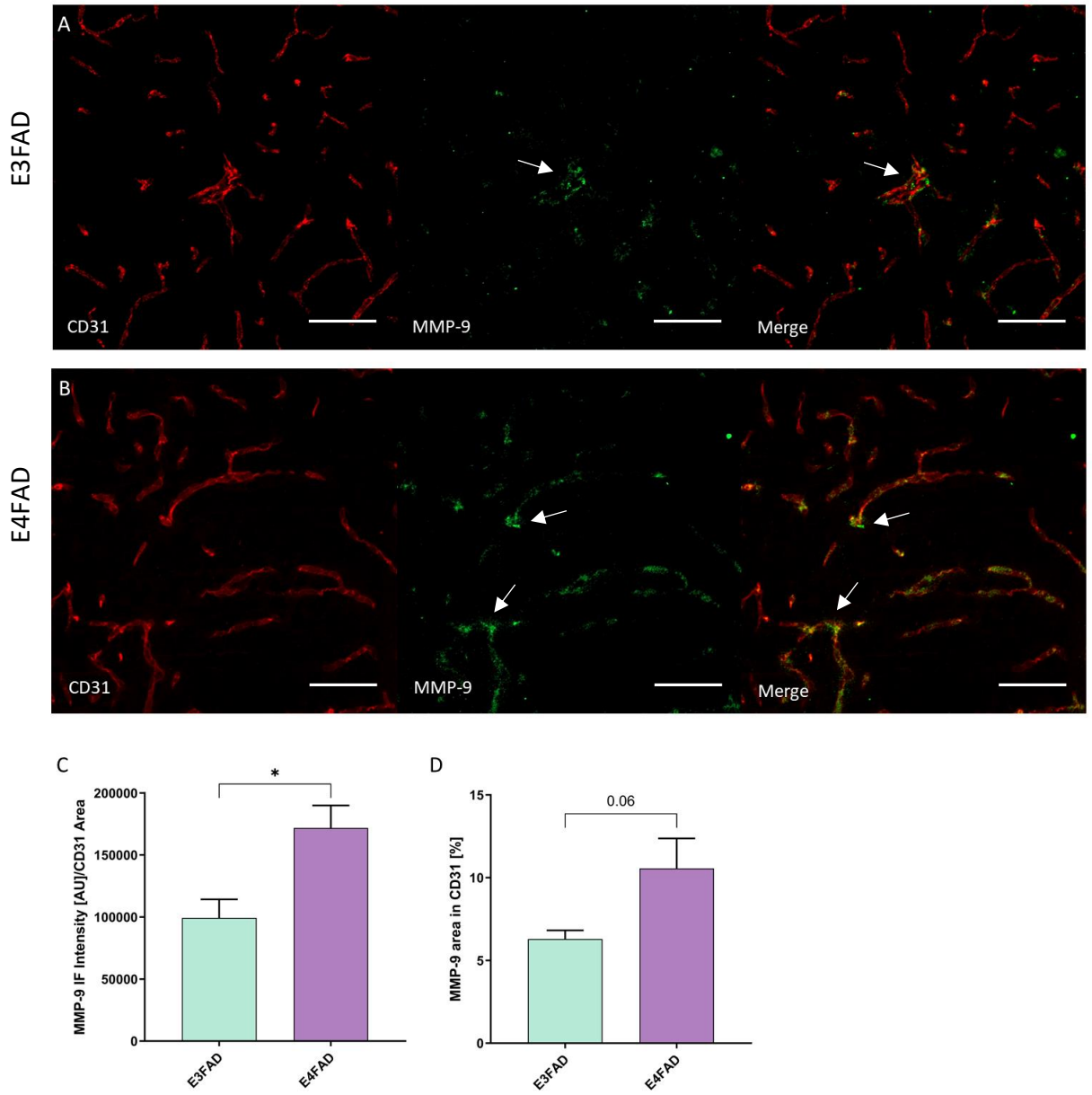


Figure 2.5: MMP-9 immunoreactivity in cortices of E3FAD and E4FAD mice.
Representative confocal images depicting 6-month-old female (A) E3FAD and (B) E4FAD mice stained with antibodies against CD31/PECAM-1 (red) and MMP-9 (green). Both E3FAD and E4FAD mice exhibited a prominent MMP-9 signal in endothelial cells surrounding blood vessels. (C) Quantification of MMP-9 immunofluorescent intensity within endothelial cells (CD31/PECAM-1). (D) Quantification of MMP-9 area within endothelial cells (CD31/PECAM-1). White arrows indicate regions of prominent MMP-9 signal. The scale bars represent 50µm. N=4. Values represent mean ± SEM. *$p<0.05$, as determined by an unpaired t-test.

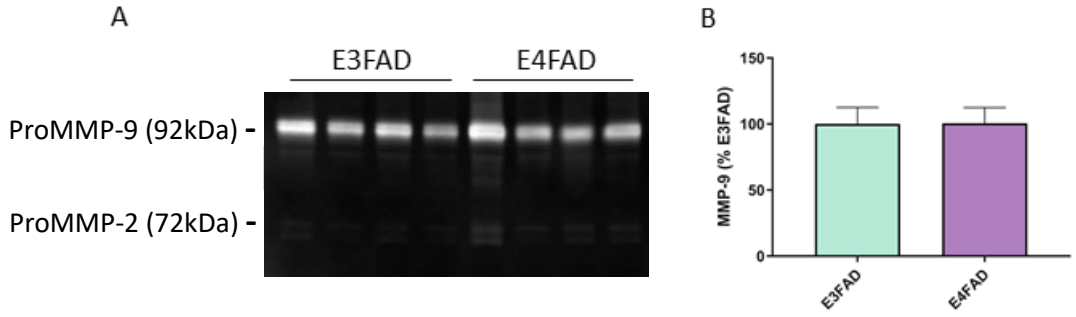


Figure 2.6: Levels of proMMP-9 in spleens of E3FAD and E4FAD mice.
Spleen samples from 6-month-old female E3FAD and E4FAD mice were analysed for MMP-9 levels using gelatin zymography. (A) Gelatin zymography demonstrating proMMP-9 bands (92kDa) and faint proMMP-2 bands (72kDa). (B) Quantification of proMMP-9 zymogram bands. Values represent mean ± SEM. N=4. No significant differences as determined by an unpaired t-test.

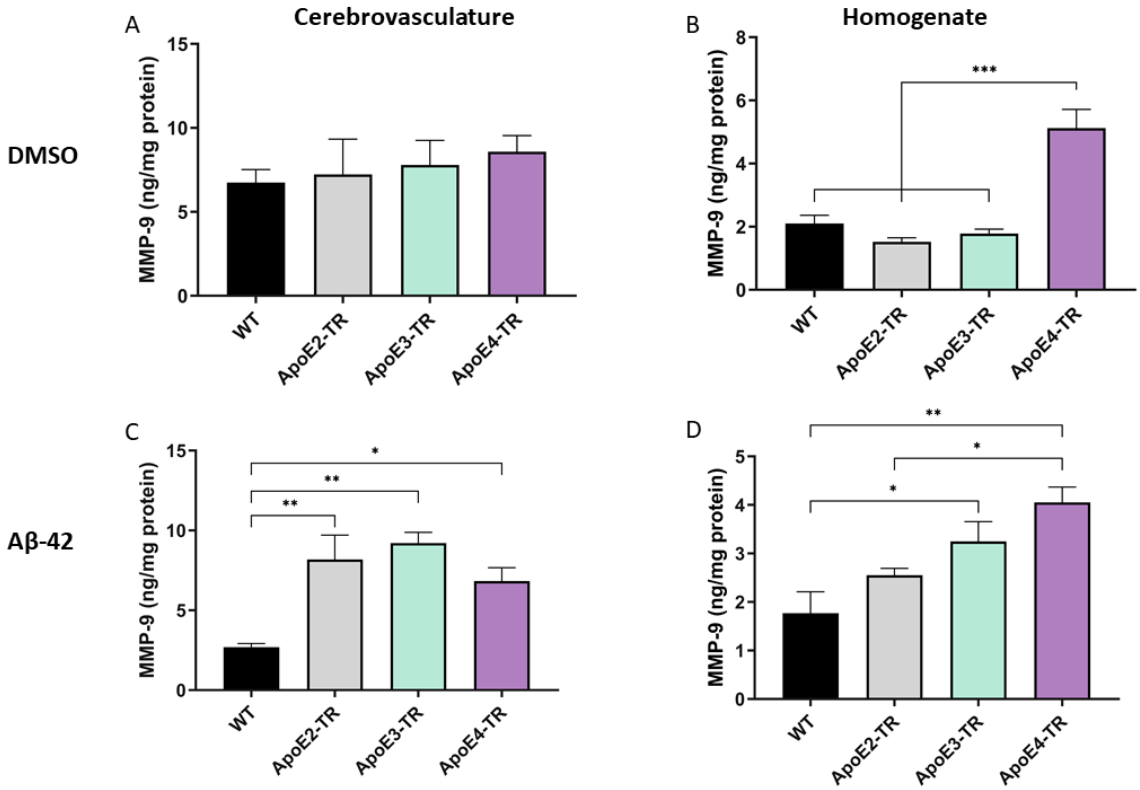


Figure 2.7: MMP-9 expression in mouse cerebrovasculature tissue from apoE-TR mice following intracranial injection with DMSO or Aβ-42.
Levels of MMP-9 were measured in the (A, C) cerebrovasculature and (B, D) whole brain homogenate of apoE2-TR, apoE3-TR, apoE4-TR, and WT mice at 6 months of age. Levels were measured following the intracranial injection of 3 µl (A, B) 100% DMSO or (C, D) Aβ-42 (1 mM in 100% DMSO). MMP-9 levels were determined using an ELISA. Values were normalised to total protein concentration and represent mean ± SEM (N = 3 per group). *p<0.05, **p<0.01, ***p<0.001 as determined by ANOVA and the BKY procedure.

2.3.3 MMP-9 expression across APOE genotype in apoE-TR mice

Whole brain homogenate and cerebrovasculature of apoE-TR mice were also analysed for MMP-9 levels. These mice had been intracranially injected with either DMSO or human Aβ-42 before euthanasia in prior studies performed by our group [182]. No changes in MMP-9 levels were detected in the cerebrovasculature of DMSO-treated mice, however global MMP-9 levels were elevated in the brain homogenate of apoE4-TR animals relative to WT, apoE2-TR and apoE3-TR mice (**$p < 0.01$) (Fig. 2.7a, b). In apoE-TR mice that had been injected intracranially with Aβ-42, elevated MMP-9 levels were detected in the cerebrovasculature of apoE2-TR, apoE3-TR and apoE4-TR mice relative to WT mice (*$p < 0.05$, **$p < 0.01$) (Fig. 2.7c). Further APOE genotype differences were also detected in the brain homogenate; MMP-9 levels were elevated in apoE3-TR mice compared to WT mice and in apoE4-TR mice relative to WT and apoE2-TR mice (*$p < 0.05$, **$p < 0.01$) (Fig. 2.7d).

2.3.4 Effect of apoE isoform on MMP-9 secretion in HBMECs and apoE-TR mice

To study MMP-9 secretion from cultured cells *in vitro*, HBMECs were treated with different lipidated apoE isoforms (previously concentrated from glia conditioned media) and either DMSO, PMA (to stimulate MMP-9 secretion) or Aβ-42 (to simulate an AD-like environment). The extracellular conditioned media from the HBMECs was examined using a quantitative substrate-based MMP-9 activity which provides a measure of endogenously active MMP-9 in addition to proMMP-9, once it has been activated on the plate by 4-aminophenylmercuric acetate (APMA). In this analysis, total MMP-9 is the combination of active and proMMP-9. ApoE alone did not appear to impact total secreted MMP-9 levels in the extracellular media (Fig. 2.8c), however upon stimulation with PMA or insult with Aβ-42, apoE isoform differences were apparent as total MMP-9 levels were higher in the presence of apoE3 and apoE4 compared to apoE2 (*$p<0.05$, **$p<0.01$) (Fig. 2.8f, i). A similar effect was observed with the levels of proMMP-9, as treatment

with apoE2 and either PMA or Aβ-42 resulted in lower levels of proMMP-9 compared to apoE3 and apoE4, with apoE4 demonstrating the highest levels (*$p<0.05$, **$p<0.01$) (Fig. 2.8e, h). Again, this effect was not observed with apoE alone (Fig. 2.8b). Lastly, in terms of the active enzyme, apoE isoform-dependent differences in active MMP-9 levels were detected when apoE was added alone and in combination with PMA. The levels of active MMP-9 were 2-fold higher following treatment with apoE3 and apoE4 relative to apoE2 (**$p<0.01$, ***$p<0.001$) (Fig. 2.8a, d).

The extracellular media was also analysed with an MMP-9 ELISA, which measures all forms of MMP-9 (i.e. proMMP-9, active MMP-9, and enzyme/inhibitor complexes). The known stimulator of MMP-9 secretion, PMA, induced the appearance of MMP-9 in the extracellular media from HBMEC cultures (Fig. 2.9a). However, MMP-9 levels in the extracellular media were below the detection limit following treatment with apoE and DMSO or apoE and Aβ-42. HBMECs treated with either apoE2, apoE3 or apoE4 in addition to PMA did not induce further MMP-9 secretion compared to control (i.e., no apoE) and no change in MMP-9 levels was apparent between apoE isoforms (Fig. 2.9a). In an *ex vivo* model, the cerebrovasculature from apoE3-TR and apoE4-TR mice was isolated and treated with DMSO, PMA or Aβ-42. Elevated MMP-9 levels were detected in the extracellular media upon treatment with Aβ-42 however, MMP-9 levels in the extracellular media were below the detection limit following treatment with PMA or DMSO. No changes were evident between the different apoE isoforms following Aβ-42 treatment (Fig. 2.9b).

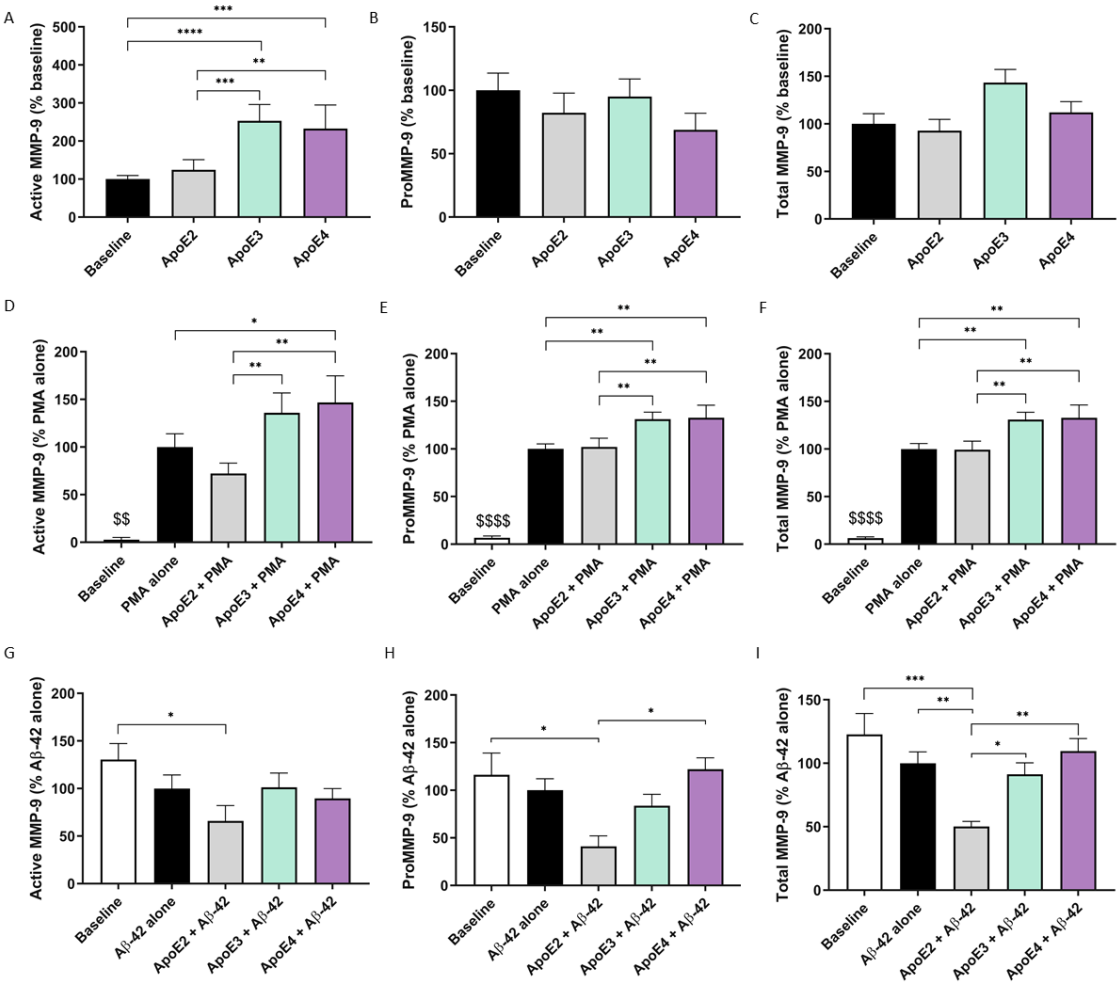


Figure 2.8: ApoE isoform differences in MMP-9 secretion in conditioned media from apoE-treated HBMECs.

Using a quantitative substrate-based MMP-9 activity assay, levels of (A, D, G) endogenously active MMP-9, (B, E, H) proMMP-9 and (C, F, I) total MMP-9 were analysed in the presence of apoE2, 3 or 4 in the conditioned extracellular media of HBMECs. Cells were stimulated with either (A, B, C) apoE alone (25 ng/ml), (D, E, F) PMA (1 μM) and apoE (25 ng/ml) or (G, H, I) Aβ-42 (2 μM) and apoE (25 ng/ml). ApoE isoform effects on secreted proMMP-9 levels and total secreted MMP-9 were apparent upon stimulation/insult. Levels of active MMP-9 were elevated in an apoE isoform-dependent manner (apoE2<apoE3<apoE4) when added alone and after stimulation with PMA. Values represent mean ± SEM. (A, B, C) n = 14, (D, E, F) n = 10, (G, H, I) n = 8. $^{\$\$}p<0.01$, $^{\$\$\$\$}p<0.0001$ indicates significant differences relative to all other conditions and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 indicates significant differences relative to indicated bars as determined by ANOVA and the BKY procedure.

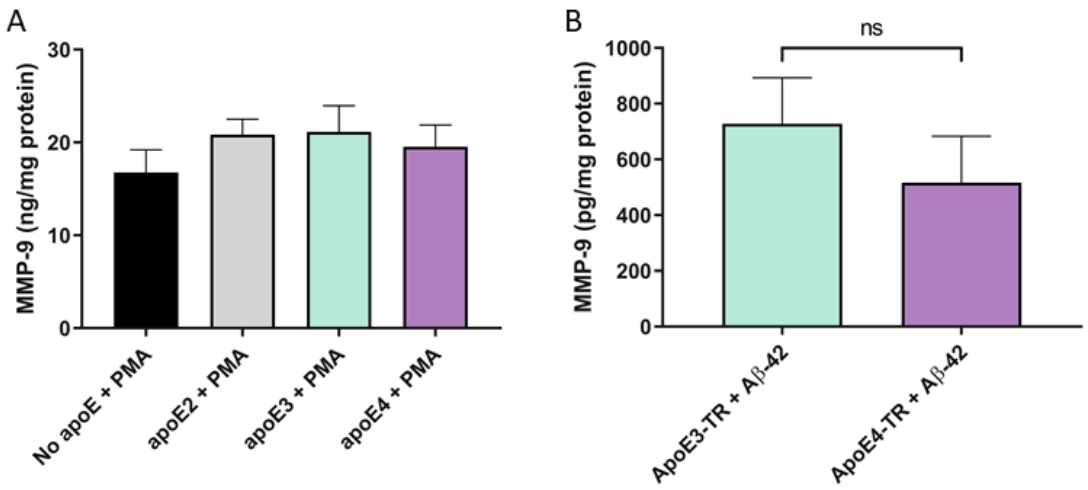


Figure 2.9: Impact of apoE isoform on MMP-9 secretion.
Secreted MMP-9 was measured in the extracellular media of A) HBMECs treated with 1 μM PMA and either apoE2, apoE3, apoE4 (25 ng/ml) or no apoE (control) and B) freshly isolated cerebrovessels from APOE targeted replacement (apoE-TR) mice treated with 2 μM Aβ-42 to stimulate secretion. No change was detected between apoE isoforms. Values represent mean ± SEM. A) N=8. No significant differences as determined by a one-way ANOVA. B) apoE3-TR: N=5, apoE4-TR: N=4. No significant differences as determined by an unpaired t-test.

2.4 Discussion

For a more thorough understanding of how APOE isoforms confer either an increased or decreased risk of developing AD, their influence on MMP-9, an enzyme previously implicated in the worsening of AD pathology [144], [200], [213], [214], [219], [220], was investigated. Previously our group reported that MMP-9 function was influenced by APOE genotype [222], however the underlying mechanisms by which APOE regulates MMP-9 remain unknown. In the present Chapter, the effect of APOE isoforms on MMP-9 expression levels and secretion from the cell (two stages at which MMP-9 can be regulated) were examined using human non-demented control and AD brain tissue, apoE mice with or without AD mutations, and human brain endothelial cells.

In this Chapter, an increase in total MMP-9 in isolated cerebrovasculature from human AD cortex samples compared to non-demented controls is reported. This observation coincides with prior reporting that MMP-9 levels were increased in AD plasma and brain parenchyma [208], [309], [335]. In addition to MMP-9 expression, cerebrovascular MMP-9 function was measured by a quantitative substrate-based activity assay. Active MMP-9 was significantly elevated in AD subjects relative to non-demented controls, indicating the changes in MMP-9 expression levels observed above were functionally active. This data corresponds with zymography and western blot experiments described by Bruno et al. (2009b) which show increased active MMP-9 levels in the middle frontal gyrus of AD samples compared to control samples. Moreover, MMP-9 activity levels have been previously reported to be increased in individuals with mild cognitive impairment, indicating that MMP-9 might be involved in early pathogenesis of AD [210]. Indeed, higher MMP-9 levels have been found alongside AD biomarkers in the CSF of cognitively healthy individuals [99], [211]. The magnitude of the expression and activity of MMP-9 secreted by brain endothelia is important as elevated levels can have detrimental effects such as disrupting BBB transport and altering Aβ clearance from the brain [144], [222].

In addition to global differences in cerebrovascular MMP-9 expression and activity between human AD and control specimens, MMP-9 levels in the AD cerebrovasculature were shown to be dependent on APOE genotype, as cerebrovessels from individuals carrying the APOE4 allele had significantly higher total MMP-9 levels than APOE4 non-carriers. This coincides with previous studies which have found that MMP-9 levels are increased in brain endothelia in AD and that APOE4 carriers showed elevated levels compared to APOE3 carriers [144]. Correspondingly, the levels of active MMP-9 in the cerebrovasculature were also higher in the APOE4 carriers with AD compared to APOE4 non-carriers with AD. ApoE isoforms have been previously found to modulate the expression and activity of certain enzymes involved in AD. For example, apoE4 but not apoE3 resulted in the reduced expression of the α-secretase, a desintegrin and metalloproteinase domain containing protein 10 (ADAM10), which is involved in the non-amyloidogenic processing of APP [336]. Moreover, in terms of function, ADAM10 activity was found to be significantly lower in apoE4 samples compared to apoE2 [337]. Overall, downregulation of ADAM10 is associated with elevated Aβ levels *in vitro* and hence implicated in the pathogenesis of AD [337], [338]. Hence, modulation of MMP-9 by apoE may have similar implications in AD pathology.

Notably, the age of onset, years of dementia, or amyloid plaque load did not correlate with MMP-9 levels in these human samples. It is also important to note the limitations of the human dataset used for these experiments as, 1) the sample size of the APOE2/2 AD group is small, due to the exceedingly low incidence of AD in APOE2 homozygotes [121], and similarly 2) the mean age of the APOE4/4 non-demented control group is younger, as APOE4/4 individuals develop AD at an earlier age than other APOE genotypes [121], making it difficult to obtain autopsied brain specimens at ages consistent with the other APOE groups. That having been said, it does not appear these factors are driving the current observations as, for example, the APOE3/4 heterozygous groups were more closely age-matched and MMP-9 levels were still significantly elevated in the APOE3/4 AD samples compared to the APOE3/4 control group and the non

APOE4 groups, suggesting an effect of APOE genotype on cerebrovascular MMP-9 levels in AD, as discussed above.

In looking more closely at the data above, it appears that neither AD or APOE4 alone are driving the increase in MMP-9, but only the combination of APOE4 and AD pathology leads to elevated MMP-9 levels in the brain. In this regard, there was no APOE genotype effect on MMP-9 levels in the non-demented samples and, likewise, in the non APOE4 groups there was no difference in MMP-9 levels between the AD and non-demented samples. Relatedly, Saarela et al. (2004) discovered an interaction between a polymorphism of the MMP-3 gene (*MMP3**5A) and the APOE4 allele which increases the overall risk of AD to a greater extent than with either allele alone. The detrimental effects of the APOE4 isoform in the progression of AD is well-defined, however the underlying mechanism has yet to be fully established. Aβ, proinflammatory cytokines and oxidative damage are elevated in AD [313] and APOE4 carriers [340]–[342] and these factors have been previously shown to influence MMP-9 levels [222], [312], [314], [315], [343]. Furthermore, oxidative stress has been linked to the activation of MMP-9 [344]. Hence, while MMP-9 levels may be mediated through these pathological features, only the combination of the AD environment and the APOE4 backdrop appears to be necessary to significantly elevate MMP-9 levels in the brain.

ApoE4 has been shown to undergo nuclear translocation, binding to double-stranded DNA with high affinity and functioning as a transcription factor, thereby directly influencing gene expression [336], [345], [346]. A recent paper by Huang and colleagues (2017) described an apoE isoform-dependent alteration of APP transcription/expression via the DLK-MKK7-ERK1/2 pathway which leads to modified Aβ production with a potency rank order of apoE4>apoE3>apoE2. Not only does this provide a reason for the increased levels of APP and Aβ in APOE4 carriers but it also indicates apoE could regulate the transcription of MMP-9 and other gene targets. ApoE has also been shown to affect protein expression levels indirectly. By stimulating the release of nitric

oxide, apoE3 but not apoE4 inhibited the TNF-α-mediated up-regulation of vascular cell adhesion molecule-1 (VCAM1), a receptor implicated in the acceleration of the aging process [348]–[350]. Theendakara et al., (2016) found that apoE4 downregulates certain protective genes which resulted in the upregulation of TNF-α, nuclear factor ϰ-light-chain-enhancer of activated B cells (NFϰB) and other inflammatory factors which have been previously implicated in the upregulation of MMP-9 [351]–[354], thus representing a more indirect mechanism by which the APOE4 genotype may impact MMP-9 expression levels.

As the above analyses were performed in autopsied human brain specimens at an advanced or end stage of AD, subsequent experiments evaluated MMP-9 levels in relation to age or disease progression in the context of APOE genotype. This was assessed in the EFAD mouse model with mice at 10, 40, and 70 weeks of age, which reflect different stages of AD-related pathology [247]. Unfortunately, due to a lack of availability of the E2FAD genotype, only E2FAD tissue at 40 weeks of age could be analysed. In the whole brain homogenate, there is an apparent age effect on MMP-9 levels between the 70-week-old and 10-week-old E4FAD mice. This is consistent with data which shows increased MMP-9 levels in age-related pathologies including AD [355] and may be due to the increased Aβ and proinflammatory cytokines which are heightened with age in EFAD mice, particularly E4FAD animals [247]–[249] and, as discussed above, have been previously shown to influence MMP-9 levels [222], [312], [314], [315], [343].

As opposed to whole brain homogenate, APOE differences were identified in the cerebrovasculature of EFAD mice. Specifically, there were significantly lower MMP-9 levels in E2FAD cerebrovessels compared to the other APOE genotypes, however no difference was evident between the E3FAD and E4FAD animals. As APOE genotype differences often manifest over time, evaluations in these animals at more advanced ages may reveal greater differences in brain MMP-9 levels across genotypes, as was demonstrated in the human brain specimens, which were obviously collected at an advanced or late stage of disease during autopsy. There are

indications of this, as a greater difference in cerebrovascular MMP-9 levels between the E4FAD and E3FAD animals was observed at 70 weeks of age compared to mice at 40 weeks of age or younger. In endothelial cells, using high-resolution laser scanning microscopy, the MMP-9 signal was particularly prominent in brain endothelial cells (CD-31/PECAM-1) surrounding blood vessels in the cortices of 6-month-old E3FAD and E4FAD mice, consistent with prior reporting [316]–[318], [356]. While no change in cerebrovascular MMP-9 expression levels was identified between E3FAD and E4FAD animals via ELISA, differences in MMP-9 immunoreactivity in the brain endothelia of these animals were apparent via confocal microscopy. This may be attributable to a greater expression of MMP-9 in endothelial cells specifically, as opposed to whole cerebrovasculature, which is comprised of various other cells, receptors, and proteins [357], [358]. Collectively, these animal studies coincide with the observations in the human brain specimens that APOE genotype can influence brain MMP-9 levels in the context of AD. The observation that APOE differences were only apparent in the cerebrovasculature, and even more specifically within the endothelial cells, draws attention to the specific regulation of MMP-9 by APOE in this brain region. MMP-9 has been shown to have detrimental effects at the BBB level in neurodegenerative diseases, for example, by increasing the ectodomain shedding of lipoprotein receptors and attenuating Aβ-42 transit out of the brain [222]. As lower levels of total MMP-9 were detected in E2FAD mice this could represent a possible mechanism whereby APOE2 delegates neuroprotection in AD [114], [119], [120], or at the very least doesn't exacerbate any ongoing pathological processes, like APOE4 may do [109], [118].

Since no APOE isoform-specific changes in MMP-9 levels were detected in the cerebrovasculature from non-demented human control samples, this was investigated further in apoE mice with no AD mutations. The small number of mice used in these studies (N = 3) should be noted and the results interpreted with caution. No changes were detected between apoE2-TR, apoE3-TR, and apoE4-TR mice, coinciding with the human studies and suggesting an AD phenotype is necessary to result in APOE-specific changes in MMP-9, at least in the cerebrovasculature. Conversely,

MMP-9 levels have been previously found to be increased in the cerebrovessels of apoE4-TR relative to apoE3-TR and apoE2-TR mouse brains [219]. This disparity may be explained by the different detection technique used to measure levels of MMP-9 in the apoE-TR mouse brains in the prior reporting, i.e. multiphoton microscopy of DQ-gelatin. Since this technique measures MMP-9 activity, it could be that the apoE isoforms may be differentially interfering with the proteolytic activity of MMP-9 and accordingly, its ability to degrade the gelatin substrate, which may be a regulatory mechanism of apoE that is independent of AD pathology. The direct influence of apoE isoforms on MMP9 function will be investigated in detail in Chapter 3.

Interestingly, when looking at the whole brain, MMP-9 levels were increased in apoE4-TR mice compared to apoE2-TR and apoE3-TR and WT mice. This may be due to the regulation of MMP-9 by APOE in specific areas in the brain other than the cerebrovasculature. Stomrud et al. (2010) found that cognitively healthy individuals with risk markers for AD development, e.g. AD-supportive CSF biomarker levels of Aβ42 or the presence of the APOE4 allele, had higher CSF MMP-9 levels compared to healthy individuals without these risk markers. Hence, there may be APOE-related changes in MMP-9 levels elsewhere in the brain which occur in the absence of AD pathology. Yet, it appears that AD pathology can still enhance these effects given that the addition of Aβ-42 (injected intracranially) in the current studies lead to further APOE differences in MMP-9 levels in whole brain homogenate (WT<apoE3-TR, apoE2-TR<apoE4-TR).

As mentioned earlier, MMP-9 is secreted as an inactive proenzyme from cells including brain endothelia [193], [316]–[318]. To determine whether the observed changes in brain MMP-9 levels could be due to alterations in the release of MMP-9 from the cell, experiments were conducted to investigate whether apoE impacts cellular MMP-9 secretion. Due to the prominent role of MMP-9 at the BBB and our prior report of MMP-9 influencing lipoprotein receptor shedding and Aβ-42 transit from the brain [222], the release of MMP-9 from cultured human brain endothelial cells and isolated cerebrovasculature from apoE-TR mice was examined. Upon stimulation with PMA

or Aβ-42, treatment with the apoE2 isoform significantly reduced MMP-9 secretion in the extracellular media relative to apoE3 or apoE4, which correlates with the findings in the E2FAD animals where cerebrovascular MMP-9 protein levels were substantially reduced versus the other APOE genotypes. In understanding the nature of these observations, the reported higher binding affinity of apoE2 for monomeric Aβ may explain the ability of apoE2 to mitigate the influence of Aβ on MMP-9 secretion, in comparison to other apoE isoforms [359], [360]. Furthermore, apoE2 has been shown to prevent the conversion of monomeric Aβ into oligomeric species [134], [361], which are known to induce MMP-9 levels to a greater extent than other forms of Aβ [362], and may further describe the findings in the E2FAD animals. In these secretion studies, an apoE isoform-specific influence on MMP-9 levels was more evident after stimulation/insult with PMA or Aβ-42. These findings are consistent with the above MMP-9 expression data in the human brains which indicate APOE isoform-mediated changes in MMP-9 levels are only apparent upon insult, e.g., AD. Moreover, these observations are consistent with a "two-hit" hypothesis in which two factors are deemed necessary to propagate disease pathogenesis, in this case AD and the APOE4 genotype driving MMP-9 levels in the brain [363]–[365].

While differences in total secreted MMP-9 (i.e. pro and active MMP-9) were observed when the extracellular media was analysed with a substrate-based activity assay, the ELISA analysis of total MMP-9 levels (i.e. proMMP-9, active MMP-9 and enzyme/inhibitor complexes) did not identify changes in MMP-9 levels between apoE isoforms. Likewise, following treatment with Aβ-42, no apoE isoform differences in the levels of total MMP-9 in the extracellular media of the isolated apoE-TR cerebrovasculature were identified. This incongruence suggests that there may be a shift in the amount of free MMP-9 vs enzyme/inhibitor complexes secreted into the media [366]. For instance, there may be more MMP-9 complexed with TIMP-1, a natural inhibitor of MMP-9, present in the apoE2-treated media, as opposed to free MMP-9. These enzyme/inhibitor complexes can be detected by the ELISA analysis but not the substrate-based activity assay. Higher MMP-9/TIMP-1 ratios and lower TIMP-1 levels have been reported in AD, however not much

is known about the effect of apoE on TIMP-1 levels [367], [368]. These findings highlight the importance of recognising methodological differences when evaluating different MMP-9 assays [362]. Alternatively, the apoE2 in the media may be directly interacting with MMP-9, inhibiting its ability to degrade the substrate in the assay. This is discussed in further detail in Chapter 3.

Collectively, the data presented in this Chapter indicates that APOE influences total MMP-9 expression levels in AD human and EFAD mouse cerebrovascular tissue and also impacts levels of pro and active MMP-9 levels in conditioned media from HBMECs. ApoE may also be affecting other aspects of MMP-9 regulation such as the conversion from proMMP-9 to active MMP-9 or MMP-9 activity directly. The effect of apoE isoforms on these regulatory processes will be investigated in Chapter 3.

Chapter 3: Functional regulation of MMP-9 by apoE: conversion, binding, colocalisation and enzymatic activity

3.1 Introduction

In Chapter 2, the levels of active MMP-9 were shown to be increased in APOE4 carriers with AD. This elevation of active MMP-9 may be a consequence of overall raised MMP-9 expression levels, but they could also be due to the influence of apoE on the regulatory mechanisms of MMP-9. In addition to regulation via protein synthesis, MMPs are known to be highly regulated once they have been secreted from the cell [318]. Post-secretional regulation of MMP-9 includes conversion of the proenzyme to active MMP-9 and inhibition of proteolytic activity. Parts of the data and conclusions presented in this Chapter have been peer-reviewed and published [319].

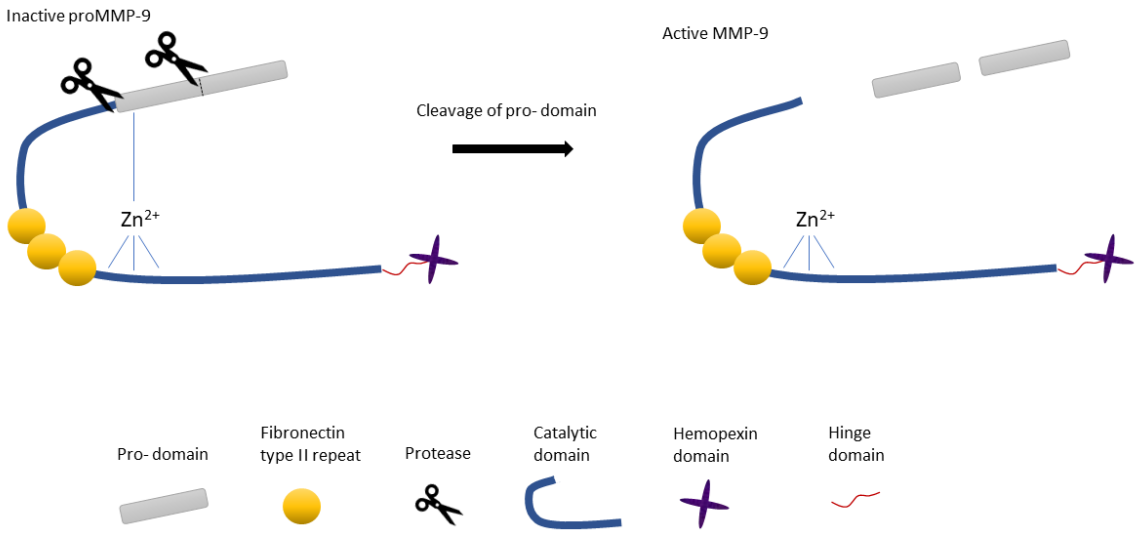


Figure 3.1: Activation of the gelatinase, MMP-9.
Like other MMPs, MMP-9 contains a pro-domain, catalytic domain, hinge region and hemopexin domain, however it also contains three fibronectin type II repeats in the catalytic domain. MMP-9 is produced as proMMP-9, a zymogen which requires activation through catalytic conversion and cleavage of the pro-domain to expose the Zn2+ dependent active site (as described by Page-McCaw et al. (2007) [197]).

The proenzyme has molecular mass of 92 kDa which contains an N-terminal pro-domain that blocks the Zn^{2+} dependent active site (Fig. 3.1) [369]. The active MMP-9 enzyme is formed by an autocatalytic conversion through a "cysteine switch" mechanism which results in the removal of a 10 kDa N-terminal pro-region [335], [370], [371]. Several activators of MMP-9 have been identified including MMP-2 [230], MMP-3 [231], MMP-7 [232], MMP-10 [233], MMP-13 [234], and the serine protease trypsin [235]. Increased MMP-9 activation is seen following inflammation [200], [372], for instance NF-$\varkappa$B signalling in human endothelial cells has been shown to affect the activation of MMP-9 [353], [373], [374]. MMP-9 is typically processed into the well-established 82-kDa active species, in some instances via an inactive 86 kDa intermediate form [231], [369], [375]. APMA, a mercurial compound and a known MMP activator, can also convert proMMP-9 to the 82 kDa active form [376]. Once activated, MMPs can be inhibited by TIMPs [377]. MMP-9 inhibitors such as TIMP1 and various synthetic inhibitors usually bind to the Zn^{2+} active site, thus preventing activity [378]. A recent paper describes an MMP-9 inhibitor which prevents the conversion of the latent proenzyme, unlike most inhibitors which target the traditional active site, thus enabling a higher specificity to MMP-9 over other MMPs [375].

As discussed in Chapters 1 and 2, APOE is a major genetic risk factor for AD. The risk of developing AD is 4- and 8-fold higher in an individual carrying one or two APOE4 alleles respectively compared to APOE3 homozygotes [109], [118]. Conversely, APOE2 is regarded as protective against AD pathology, reducing risk by nearly half [114], [119], [120]. While considerable research has been conducted to define the detrimental effects of the APOE4 isoform that confer this heightened risk, the picture is incomplete. Each of the three major isoforms of APOE differ only at one or two positions; while APOE3 has cysteine at position 112 and arginine at position 158, APOE2 has cysteine at both positions and APOE4 has arginine at both positions [111]. This drastically alters the structure and function of the apoE proteins and can affect their binding abilities. For instance, the apoE isoforms differ in their binding affinities to LDLR; apoE3 and apoE4 bind to LDLR with high affinity whereas apoE2 binding was not as strong [379], [380].

Given that many MMP-9 inhibitors function through binding to MMP-9 in some way [375], experiments were conducted to assess if apoE could bind to MMP-9 and whether the apoE isoforms would exhibit different binding affinities. Determining if the apoE isoforms can bind to MMP-9 will not only indicate whether they can interact physiologically, but also give an indication as to whether apoE would be able to modulate MMP-9 directly. In this Chapter, the direct interactions between apoE and MMP-9 were investigated through binding association studies and localisation analyses in the brains of EFAD mice. In addition, the potential consequences of these interactions, namely the effect of apoE on MMP-9 conversion and activity, were determined.

Investigating apoE behaviour in both its lipid-free and lipid-bound state is necessary to improve our understanding of its function in the context of AD pathology as apoE lipidation status has been shown to have an effect on its interactions, for instance with Aβ and its consequent aggregation, deposition and clearance [125], [381]–[384]. Therefore, apoE lipidation status was considered when studying the binding of apoE to MMP-9 and the resulting impact on MMP-9 conversion to its active form.

3.2 Materials and methods

3.2.1 Materials

APMA, recombinant human MMP-9, and dextran were purchased from MilliporeSigma (St. Louis, MO, USA). Recombinant apoE was purchased from BioVision (Milpitas, CA). ELISA kits for LRP1 and LDLR were purchased from Cedarlane Labs (Burlington, NC, USA). ELISA kits for human apoE were purchased from MBL International (Woburn, MA). Halt enzyme inhibitor cocktails, the BCA protein assay and HBSS were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

3.2.2 Human cortex samples

Human cortex samples (inferior frontal gyrus) were obtained from the autopsied brains of AD and non-demented subjects with different APOE genotypes as described in Chapter 2.2.2.

3.2.3 Animals

ApoE-TR mice and EFAD mice were used in the present studies. A description of these models and the housing conditions of the animals is presented in Chapter 2.2.3.

3.2.4 Isolation of brain fractions

Frozen human cortex samples and mouse brains were homogenised and the cerebrovasculature was isolated using a step-wise density gradient extraction process as previously described [182] and as summarised in Chapter 2.2.4. Following the separation of the cerebrovasculature, the remaining parenchyma and soluble brain fraction were centrifuged for a further 10 minutes to separate these two fractions. The parenchyma was resuspended in HBSS and centrifuged for a final 5 minutes before the pellet was collected in lysis buffer. All fractions were stored at -80 °C prior to analysis.

3.2.5 Collection and enrichment of human lipidated apoE

The mixed glial cultures were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). Cortical glial cultures were prepared from apoE2-TR, apoE3-TR, or apoE4-TR mice as previously described [332] and as summarised in Chapter 2.2.8.

3.2.6 Artificial lipidation of apoE

Recombinant apoE was artificially lipidated as previously described using a protocol adapted from prior reporting [385]. Liposome preparation: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, Alabaster, AL) and un-esterified cholesterol (Avanti Polar Lipids, Alabaster, AL) were mixed in a glass vial at a molar ratio of 90:5. This ratio was selected to mimic the physiological lipid composition of high-density lipoprotein (HDL)-like apoE particles. Lipids were resuspended in PBS at a concentration of 5 μg lipids/μl. The solution was mixed thoroughly by vortexing intermittently for 5-10 min (with 1-2 min intervals) to produce liposomes. Complete hydration of liposomes was achieved by incubating the solution at room temperature for 30 min and occasional vortex mixing.

ApoE lipidation: sodium cholate (50 mg/ml, Sigma-Aldrich, St. Louis, MO) was slowly titrated into the liposome solution (2-3 volumes of sodium cholate for 1 volume of lipids). The solution turbidity cleared after 5 min of gentle vortex mixing (1 min interval) and the preparation was kept at room temperature for 30-60 min. Reconstituted apoE was then added to the liposome preparation (apoE:POPC:cholesterol, molar ratio of 1:90:5) and mixed gently for 5-10 min (1-2 min interval). The solution was kept at room temperature for 1 h and dialysed (10 kDa cut-off membrane) against PBS for 4 h at room temperature (to promote removal of detergents), followed by 60-72 h at 4 °C. Samples were desalted and small molecular weight solutes were removed using PD-10 Desalting Columns (Sigma-Aldrich, St. Louis, MO).

3.2.7 Zymographic analysis of the impact of apoE on MMP-9 conversion to the active form

Recombinant human MMP-9 (1 μg/ml) was incubated with recombinant, artificially lipidated, or glia-lipidated apoE2, apoE3 or apoE4 (25 ng/ml) for 45 minutes at 37 °C before being incubated with APMA (1 mM) for 45 minutes at 37 °C. Samples were then analysed by gelatin zymography to determine pro and active MMP-9 levels. For each sample, a total of 7.5 ng of total protein was loaded. The samples were separated on a 10% precast polyacrylamide gel with gelatin (Thermo Fisher Scientific, Waltham, MA) and developed as described in Chapter 2.2.7.

3.2.8 Cell-free activity assay

The effect of apoE isoforms on MMP-9 activity was assessed in a cell-free paradigm utilising a fluorescent substrate as per the manufacturers' instructions (Anaspec, USA). Briefly, recombinant MMP-9 (5 nM) was incubated in the presence of lipidated apoE2, 3 and 4 (0-250 ng/ml) (collected from mixed glial cultures, described in Chapter 3.2.5) or SB-3CT (2-[[(4-Phenoxyphenyl)sulfonyl]methyl]thiirane) (1 μM) and the fluorescent substrate for 1 hour at 37 °C before detection of fluorescence. To determine whether the substrate was metabolised by apoE itself, fluorescent substrate was incubated with differing concentrations of apoE alone in the absence of MMP-9. These values were used as background controls for each apoE isoform-MMP-9 treatment combination. Fluorescence was measured at 340/490 nm excitation/emission wavelengths using a BioTek Synergy HT microplate reader.

3.2.9 Binding studies

The binding of MMP-9 to either recombinant apoE2, 3 and 4 or artificially lipidated apoE3 was evaluated using an Octet RED96 instrument equipped with Streptavidin biosensors purchased from ForteBio (Menlo Park, CA). Biotinylated recombinant human active MMP-9 in PBS (2 μg/ml) was immobilised to the streptavidin biosensors for 1800 seconds and washed three times for 60 seconds in PBS at 1,000 rpm using the Octet platform. The Octet analysis was performed

at room temperature under a continuous agitation at 1,000 rpm. Each of the apoE isoforms were then loaded at multiple concentrations (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml) to separate sensors then allowed to dissociate in PBS (apoE2 association time: 1200s, dissociation time: 1200s, apoE3 association time: 1800s, dissociation time: 3800s, apoE4 association time: 1200s, dissociation time: 1200s). Individual binding affinities were assessed by measuring the dissociation constant (KD) determined from the steady state.

3.2.10 Tissue processing

All mice were humanely euthanised, and their brains were collected and fixed in 4% paraformaldehyde for 48 h as previously described [334] and as summarised in Chapter 2.2.11.

3.2.11 Immunofluorescence

The protocol used for immunofluorescence staining is described in Chapter 2.2.12. Briefly, tissue sections were deparaffinised, rehydrated, and subjected to antigen retrieval for 7 min in glycine buffer (pH 3) at 100 °C before blocking in 100% SeaBlock (EastCoast Bio, MA, USA), 0.5mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 for 1 h. Sections were incubated in PBS containing 10% SeaBlock, 0.33mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 and the respective panel of primary antibodies overnight at 4 °C. The following antibodies were used: ab38898 (anti-MMP-9, 1:200, Abcam, Cambridge, MA, USA), ab1907 (anti-apoE [E6D7], 1:200, Abcam, Cambridge, MA, USA), AF3628 (anti-CD31/PECAM-1, 1:1000, Novus Biologicals, Centennial, CO, USA). After three washing steps in PBS for 5 min, sections were incubated in PBS containing 10% SeaBlock, 0.33mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 and the respective panel of secondary antibodies for 1 h in the dark at room temperature in a humidified chamber. The following secondary antibodies were used: donkey anti-rabbit, anti-goat, anti-mouse conjugated to Alexa 488, 568 and 647, respectively (1:1000, Life technologies). For qualitative analysis of the colocalisation between MMP-9 and apoE, orthogonal projections of cortical brain regions from E3FAD and E4FAD mice (n = 4 for each genotype, all female mice, 6 month of age) were

analysed. Six images were generated for each mouse. Averages calculated from the six images were used for statistical analysis.

3.2.12 Statistical analysis

Data are expressed as mean ± SEM. Data was checked for normality and statistical significance was determined by ANOVA followed by the two-stage step-up method of Benjamini, Krieger and Yekutieli unless otherwise stated. A p-value lower than 0.05 was used to indicate a statistically significant difference. Statistical analyses were performed with GraphPad Prism 8.

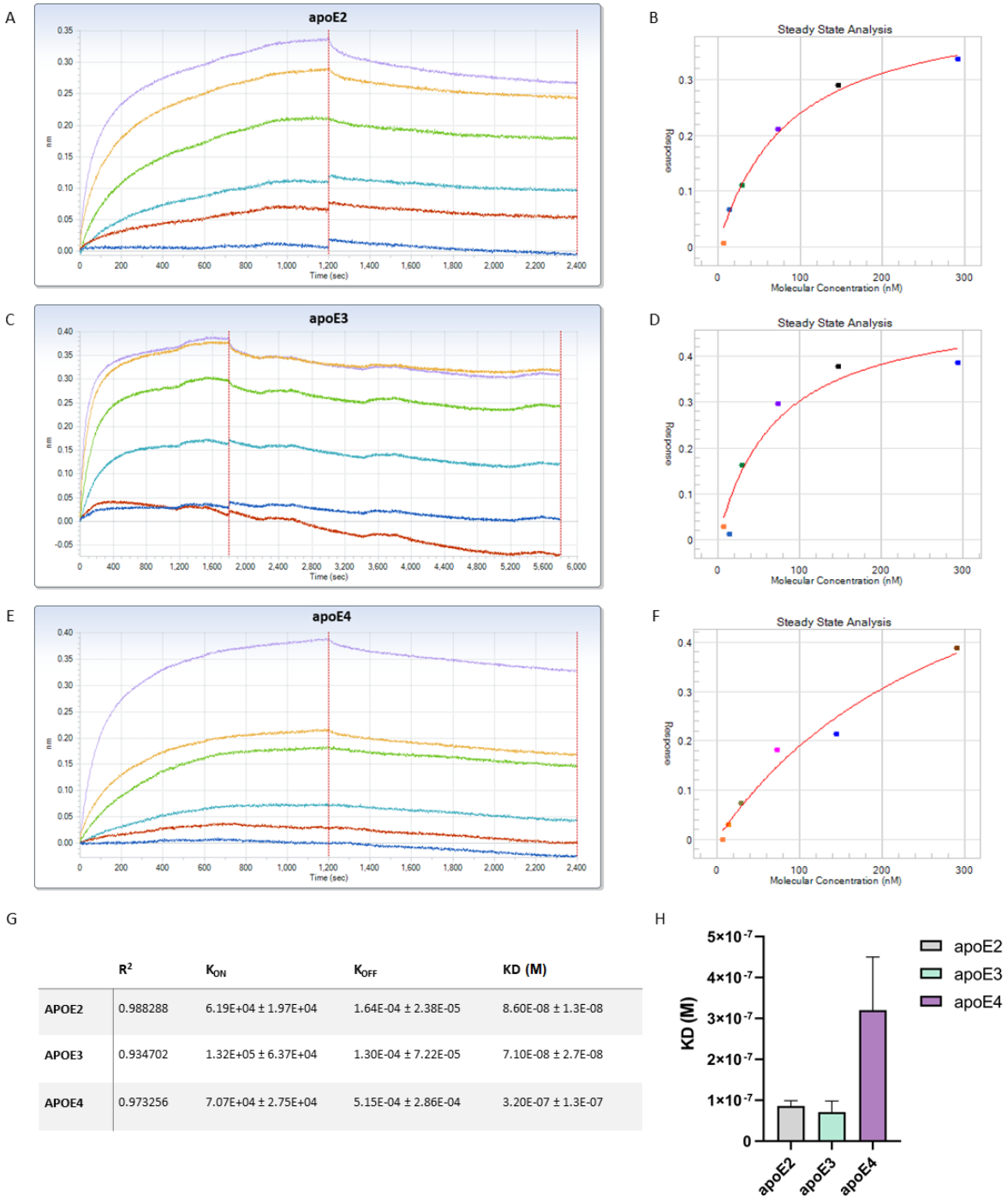
3.3 Results

3.3.1 Binding interactions between apoE and MMP-9

Kinetic binding studies using biolayer interferometry were conducted using the ForteBio Octet RED96 instrument. Active MMP-9 was able to bind to all isoforms of recombinant apoE in the association step (Fig. 3.2a, c, e), but the subsequent dissociation in PBS was limited, even when the dissociation time was extended, as shown with the apoE3 isoform (Fig. 3.2c). The dissociation constant (KD) was measured from the steady state analysis for each isoform (Fig. 3.2b, d, f). ApoE4 displayed a larger KD than both apoE3 and apoE2 (4.5-fold and 3.7-fold higher respectively) signifying a weaker binding interaction between MMP-9 and apoE4 compared to the other apoE isoforms (Fig. 3.2g, h). Artificially lipidated apoE3 did not bind to MMP-9 (Fig. 3.3).

3.3.2 Effect of apoE isoform on pro MMP-9 conversion to activated MMP-9

In assessing the effect of apoE isoforms on the conversion of proMMP-9 to the active form of MMP-9, as anticipated, both proMMP-9 (92 kDa) and active MMP-9 (82 kDa) bands could be detected using zymography following treatment of recombinant proMMP-9 with APMA (Fig. 3.4a, c, e), which has been previously shown to convert proMMP-9 into its active form [335], [370]. With respect to apoE, conversion of MMP-9 was increased by 25% and 62% in the presence of recombinant apoE4 compared to apoE2 and apoE3 respectively, following APMA activation (***$p<0.001$) (Fig. 3.4a, b). However, following incubation of MMP-9 with glia-lipidated apoE, the greatest conversion of proMMP-9 to active MMP-9 was observed in the presence of apoE2 (Fig. 3.4c, d). Lastly, concerning apoE that had been artificially lipidated, no differences between apoE isoforms were identified (Fig. 3.4e, f).



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	R^2	K_{ON}	K_{OFF}	KD (M)
APOE2	0.988288	6.19E+04 ± 1.97E+04	1.64E-04 ± 2.38E-05	8.60E-08 ± 1.3E-08
APOE3	0.934702	1.32E+05 ± 6.37E+04	1.30E-04 ± 7.22E-05	7.10E-08 ± 2.7E-08
APOE4	0.973256	7.07E+04 ± 2.75E+04	5.15E-04 ± 2.86E-04	3.20E-07 ± 1.3E-07

Figure 3.2: Kinetic binding studies of MMP-9 and apoE2, apoE3 or apoE4 using Bio-Layer Interferometry.
Biotinylated recombinant human MMP-9 was immobilised to streptavidin biosensors (1800 seconds). Recombinant apoE was then added in increasing concentrations (dark blue: 0.25 µg/ml, red: 0.5 µg/ml, light blue: 1 µg/ml, green: 2.5 µg/ml, orange: 5 µg/ml, purple: 10 µg/ml) to separate sensors then allowed to dissociate in PBS (A: apoE2. Association time: 1200s, Dissociation time: 1200s, B: apoE3. Association time: 1800s, Dissociation time: 4000s, C: apoE4. Association time: 1200s, Dissociation time: 1200s). Individual binding affinities were assessed by measuring the dissociation constant (KD) (G, H) determined from the steady state (D: apoE2, E: apoE3, F: apoE4). ApoE4 demonstrated the weakest binding affinity; the KD for apoE4 was greater compared to apoE3 and apoE2. Values represent mean ± SEM. N=5-6.

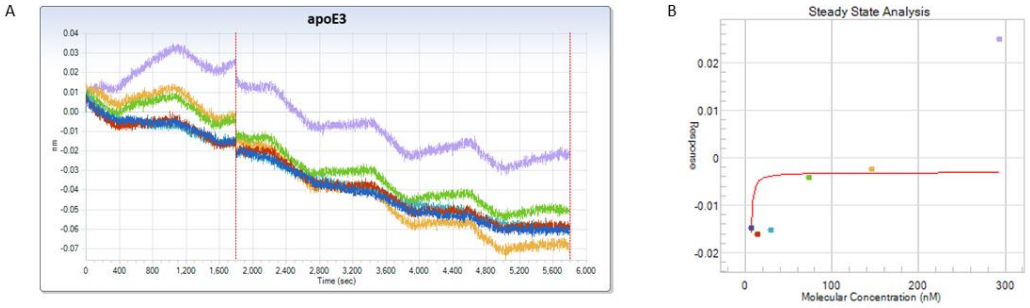

Figure 3.3: Figure 14: Kinetic binding studies of MMP-9 and artificially lipidated apoE3 using Bio-Layer Interferometry.
Biotinylated recombinant human MMP-9 was immobilised to streptavidin biosensors (1800 seconds). (A) Artificially lipidated apoE3 was then added in increasing concentrations (dark blue: 0.25 μg/ml, red: 0.5 μg/ml, light blue: 1 μg/ml, green: 2.5 μg/ml, orange: 5 μg/ml, purple: 10 μg/ml) to separate sensors then allowed to dissociate in PBS (Association time: 1800s, Dissociation time: 4000s). (B) Steady state analysis showed limited binding of MMP-9 to artificially lipidated apoE3.

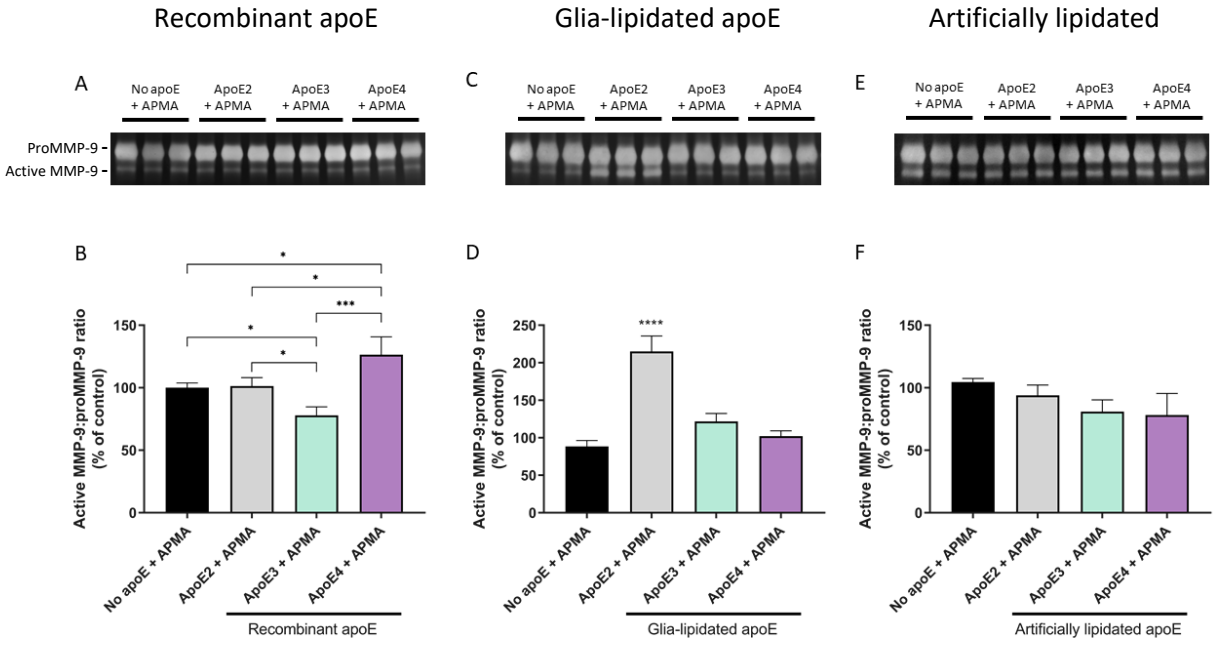

Figure 3.4: Effect of apoE isoform on the conversion of MMP-9 to the active form.
Recombinant human MMP-9 was incubated with 25 ng/ml (A, B) recombinant (C, D) glia-lipidated (GCM) or (E, F) artificially lipidated recombinant human apoE2, 3 or 4 or no apoE (control) before being activated with APMA (1 mM). (A, C, E) Gelatin zymography demonstrating pro and active MMP-9 bands. (B, D, F) Quantification of zymogram bands (ratio of active to proMMP-9). (B) n = 17, (D) n = 13, (F) n = 5-6. Values represent mean ± SEM. *p<0.05, ***p<0.001, ****P<0.0001 as determined by a one-way ANOVA and the BKY procedure.

3.3.3 Effect of apoE isoform on MMP-9 activity

Using a cell-free MMP-9 activity assay, each apoE isoform significantly attenuated MMP-9 activity in a dose-dependent manner compared to control conditions (MMP-9 alone) (Fig. 3.5). Furthermore, apoE isoform-specific differences in modulating MMP-9 activity were observed at each apoE concentration on the order of apoE2>apoE3>apoE4. However, while apoE4 did impact MMP-9 function, apoE2 and apoE3 were considerably more effective than apoE4 in modulating MMP-9 activity. At 250 ng/ml apoE2 and apoE3 resulted in an 82% and 77% reduction in MMP-9 activity with respect to control, respectively, compared to a 48% reduction by apoE4, which equates to a 3-fold difference between apoE2 and apoE4 at this concentration (****$p<0.0001$).

3.3.4 Localisation of apoE and MMP-9 in brain endothelia

Having shown that MMP-9 can directly bind to apoE in a cell-free environment, experiments were conducted to investigate this interaction in a physiological setting by using immunofluorescence and confocal microscopy. Brain cortices of 6-month-old E3FAD and E4FAD mice were analysed for MMP-9 immunoreactivity in cortical endothelial cells (Fig. 3.6a, b). A high proportion of apoE was confined to amyloid plaques leading to higher overall levels of apoE in E4FAD mice compared to E3FAD (Fig. 3.6e). When apoE confined to amyloid plaques was excluded from the analysis, levels of apoE were no different between E3FAD and E4FAD mice (Fig. 3.6f). While levels of CD-31 or plaque-free apoE did not differ between APOE genotypes (Fig. 3.6f, h), there was a 56% increase in MMP-9 immunoreactivity in cortical endothelial cells from E4FAD mice compared to E3FAD mice, as discussed in Chapter 2.4 (*$p<0.05$) (Fig. 3.6c). Importantly, there were several areas where MMP-9 and apoE predominantly overlapped (indicated by white arrows) and interaction was significantly higher in E3FAD mouse cortices relative to E4FAD (*$p<0.05$) (Fig. 3.6g).

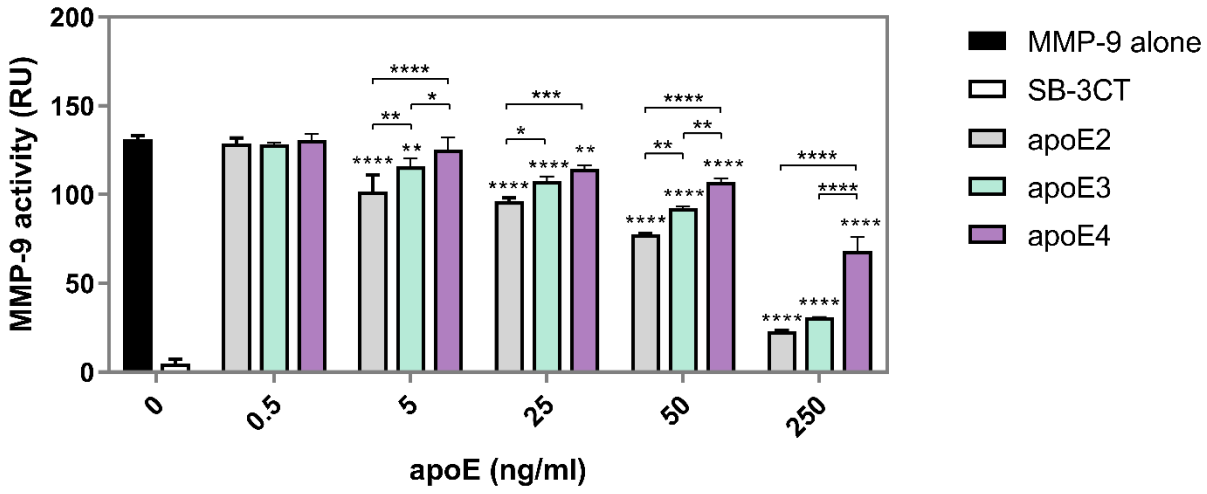

Figure 3.5: Differential modulation of MMP-9 activity by apoE isoforms.
MMP-9 activity was significantly modulated by apoE in an isoform and dose-dependent manner (apoE2>apoE3>apoE4). Differences between each of the apoE isoforms were statistically significant at concentrations ≥ 5 ng/ml. Treatment of 1 μM SB-3CT was included as a positive control and showed considerable attenuation of MMP-9 activity. Values represent mean ± SEM (N = 3) and are expressed as fluorescent units. Statistical significance was determined by two-way ANOVA followed by the BKY procedure. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Asterisk symbols above bars indicates significant differences compared to MMP-9 alone. Asterisk symbols above brackets indicates significant differences from other apoE isoforms at that concentration.

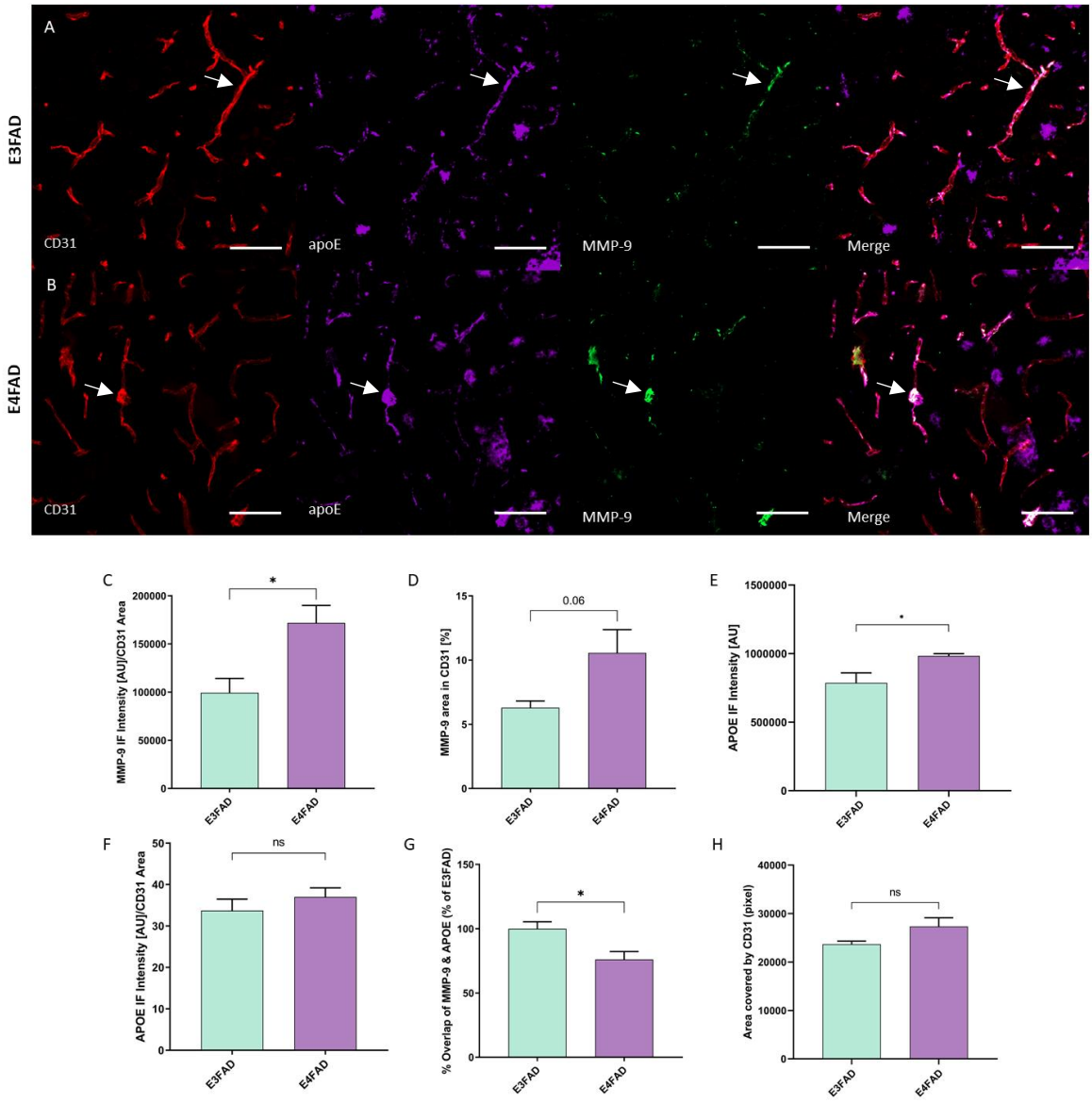

Figure 3.6: MMP-9 and apoE immunoreactivities in cortices of E3FAD and E4FAD mice.
Representative confocal images depicting 6-month-old female (A) E3FAD and (B) E4FAD mice stained with antibodies against CD31/PECAM-1 (red), apoE (E6D7, purple) MMP-9 (green). Both E3FAD and E4FAD mice exhibited a prominent MMP-9 signal that overlapped with apoE in endothelial cells surrounding blood vessels (indicated by white arrows). (C) Quantification of MMP-9 immunofluorescent intensity within endothelial cells (CD31/PECAM-1). (D) Quantification of MMP-9 area within endothelial cells (CD31/PECAM-1). (E) Quantification of total apoE immunofluorescent intensity. (F) Quantification of apoE immunofluorescent intensity within endothelial cells. (G) Percentage overlap of MMP-9 and apoE areas normalised to total MMP-9 area. (H) Quantification of the area covered by endothelial cells (CD31/PECAM-1). The scale bars represent 50µm. N=4. Values represent mean ± SEM. *$p<0.05$, as determined by unpaired t-tests.

3.3.5 Differences in lipoprotein receptor levels in human brain tissue

LRP1 levels were analysed in the soluble brain fraction, cerebrovasculature and whole brain homogenate of human AD and non-demented control subjects (Fig. 3.7). Levels of LRP1 were decreased in the whole brain homogenate and cerebrovasculature of AD subjects and further differences were detected across APOE genotype (APOE4>APOE3>APOE2) (Fig. 3.7a, b, e, f). No changes in LRP1 levels were observed in the soluble brain fraction (Fig. 3.7c, d). These results are unpublished data from our team and derived from the dissertation thesis of Dr. Ben Shackleton.

3.3.6 Differences in lipoprotein receptor levels in EFAD mouse brain

The levels of the lipoprotein receptors LRP1 and LDLR were analysed in the soluble brain fraction, cerebrovasculature and whole brain homogenate of E3FAD and E4FAD mice at 40 and 70 weeks of age (Fig. 3.8). Levels of LDLR in the cerebrovasculature were increased in E4FAD mice relative to E3FAD mice at 40 weeks of age but then were decreased in both genotypes at 70 weeks of age (Fig. 3.8b). While the levels of LDLR did not differ between APOE genotypes or by age in the soluble brain fraction or the whole brain homogenate (Fig. 3.8a, c), levels of LRP1 were elevated in the soluble brain fraction of E4FAD mice at 70 weeks of age compared to 40 weeks of age (Fig. 3.8d). However, there were no differences in LRP1 levels in the cerebrovasculature or the whole brain homogenate in either E3FAD or E4FAD mice (Fig. 3.8e, f).

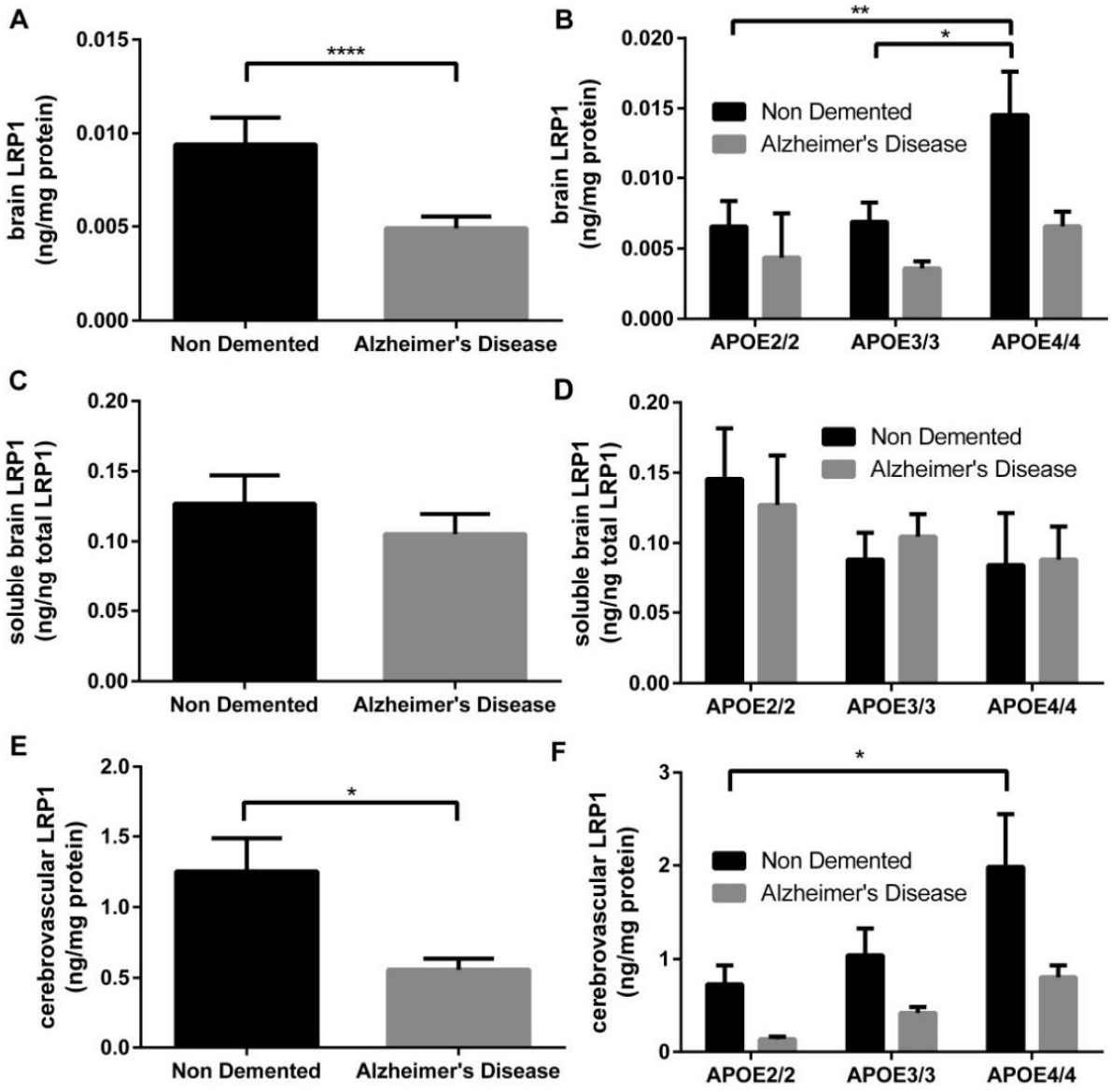

Figure 3.7: Expression of LRP1 in human cortex samples.
(A) Expression of LRP1 in the total homogenate was decreased in AD vs non-demented control subjects. (B) This was APOE genotype-dependent; non-demented APOE4/4 individuals displayed increased LRP1 levels relative to non-demented APOE3/3 and APOE2/2 subjects and compared to APOE4/4 AD subjects. No changes in LRP1 expression were detected between (C) AD and control subjects nor with regards to (D) APOE genotype. (E) Expression of LRP1 in the cerebrovasculature was decreased in AD vs non-demented control subjects. (F) This was also APOE genotype-dependent; non-demented APOE4/4 individuals displayed increased LRP1 levels relative to non-demented APOE2/2 subjects and compared to APOE4/4 AD subjects. Values represent mean ± SEM (N = 2 for AD APOE2/2 subjects, N = 10 for all other genotype/disease groups). Statistical significance was determined by two-way ANOVA followed by Tukey's post-hoc analysis. *$p<0.05$, **$p<0.01$, ****$p<0.0001$. These results are unpublished data from our group and derived from the dissertation thesis of Dr. Ben Shackleton.

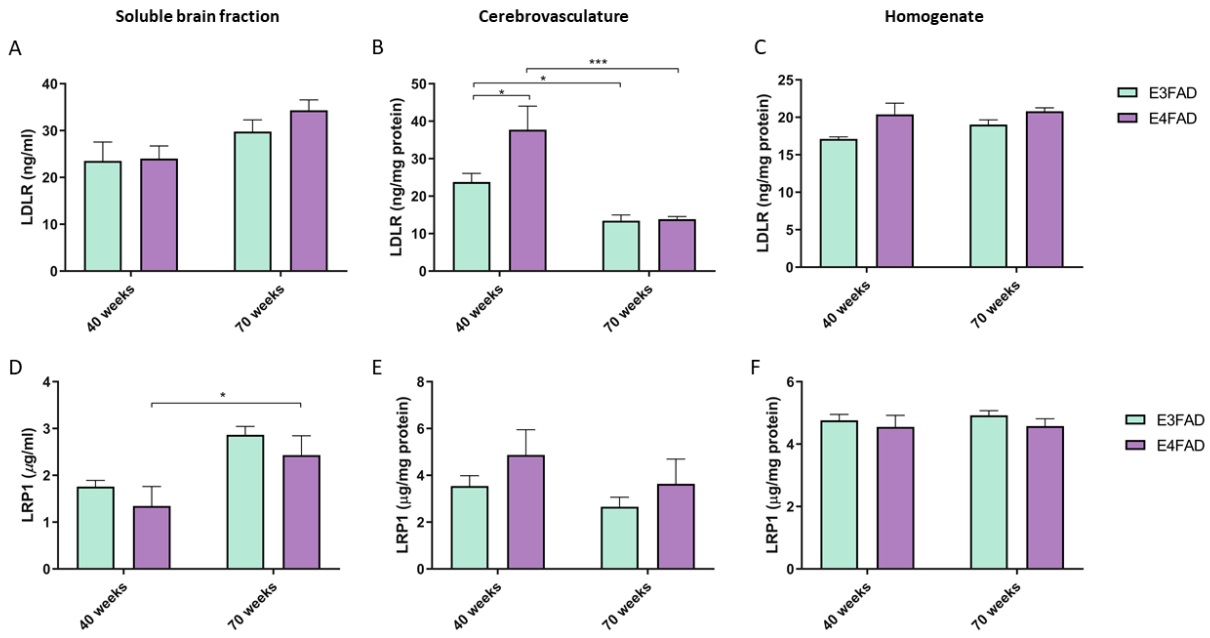

Figure 3.8: Expression of LRP1 and LDLR in 40-week-old and 70-week-old EFAD mouse brain. Expression of LDLR was not different in E3FAD compared to E4FAD mice or in 40-week-old compared to 70-week-old mice in (A) the soluble brain fraction or (C) the whole brain homogenate. (B) Levels of LDLR were increased in E4FAD relative to E3FAD mice in the cerebrovasculature of 40-week-old mice and both genotypes showed decreased levels in 70-week-old mice. Expression of LRP1 was not different in E3FAD compared to E4FAD mice or in 40-week-old compared to 70-week-old mice in (E) the cerebrovasculature or (F) the whole brain homogenate. (D) Levels of LRP1 were increased in E4FAD at 70 weeks of age compared to 40 weeks of age. Values represent mean ± SEM (N = 4). Statistical significance was determined by two-way ANOVA followed by the BKY procedure. *$p<0.05$, ***$p<0.001$.

3.4 Discussion

MMP-9 undergoes extensive regulation following secretion from the cell [318]. Expressed as a proenzyme, it must undergo proteolytic cleavage before becoming active [228], [229]. Inhibitors of MMP-9 have been shown to be able to prevent this cleavage and thus modulate the activation of MMP-9 [375]. Moreover, once in its active form, MMP-9 activity can be inhibited by both endogenous and synthetic inhibitors [378]. In this Chapter, apoE was shown to impact the conversion of MMP-9 to its active form and in addition have a direct effect on its activity. Furthermore, there were isoform-specific differences in this regulation.

Since the discovery that the APOE4 allele is a high-risk genetic factor for AD, researchers have been investigating the mechanisms underlying its detrimental effects through the use of recombinant or delipidated apoE, which provide a relatively uncomplicated means of assessing apoE function [386]. To obtain a direct measure of the influence of apoE on the conversion of proMMP-9 to the active form, proMMP-9 was incubated with APMA (a known MMP-9 activator) in the presence of each apoE isoform [335], [370]. The incubation of proMMP-9 with recombinant apoE4 resulted in a greater degree of conversion relative to apoE2 and apoE3, suggesting that apoE may regulate MMP-9 disposition by modulating proMMP-9 conversion to the active enzyme. Biolayer interferometry experiments demonstrated that the binding affinity of recombinant apoE4 to MMP-9 was 3-times weaker than the other isoforms, which may describe the enhanced conversion of proMMP-9 to active MMP-9 in the presence of apoE4. In other words, apoE4 may be less able to modulate the catalytic cleavage of proMMP-9 to active MMP-9, which has previously been shown for other proteins [235], [375], [387].

While recombinant apoE has its advantages, lipidation status has been shown to influence the degree to which apoE binds to receptors and other proteins [383], [388]. In the current studies, artificially lipidated apoE, generated by using a ratio of cholesterol and POPC selected to mimic the physiological lipid composition of HDL-like apoE particles [385], was used to investigate the

potential differences in binding affinity that may occur following lipidation of apoE, However, limited binding was observed between MMP-9 and this apoE species. While this is a common approach used by laboratories to generate lipidated apoE, the method used to artificially lipidate apoE may not be reflective of the natural lipid composition and could have resulted in the binding sites being blocked by the lipid particles. Correspondingly, as a lack of binding seemingly occurred between MMP-9 and these artificially lipidated apoE species, this could describe why no changes were detected between any of the artificially lipidated apoE isoforms when these species were incubated with MMP-9 and APMA in the conversion studies.

While these studies with recombinant apoE, both delipidated and artificially lipidated, provide important information regarding apoE in the context of AD, they may not be completely relevant to physiological conditions inside the brain. ApoE produced in the brain is expressed primarily by glia [112], [389]–[392] so using human apoE generated from the mixed glial cultures of apoE-TR mice in these studies provided a more physiologically relevant source of lipidated apoE by which to examine the effect of apoE on MMP-9 regulation. Interestingly, a significantly higher degree of conversion was observed in the presence of the glia-lipidated apoE2 compared to apoE3 and apoE4. A limitation of this study is that the glia-generated lipidated apoE was collected from conditioned media, so there may be confounding influences from other glial-secreted proteins in these preparations which would need to be determined through further analysis of the glia conditioned media. For example, apoE4 is associated with an increased pro-inflammatory response compared to apoE2 and apoE3, leading to elevated levels of cytokines [138], [393], [394], though, this has been shown to vary depending on cell type; the release of pro-inflammatory cytokines (TNF-α, nitric oxide, IL-1β, or IL-6) from microglia after lipopolysaccharide stimulation follows the pattern (apoE4>apoE3>apoE2), but this order is reversed with astrocytes [395]–[397]. However, in cells that have not been activated by lipopolysaccharide or other methods of stimulation, baseline levels of cytokines in the media are often too low to be detected [138], [395], [397]. Therefore, while it's possible that increased proinflammatory factors in the conditioned

media from apoE2-TR mixed glial cultures, such as increased NFκB levels, is impacting MMP-9 conversion levels [353], [373], [374], they are often in such low abundance in the extracellular media under non-stimulated conditions that their potential influence is likely negligible [138], [395], [397]. It should also be noted that the glia-lipidated apoE2 was sourced from apoE2 homozygous mice, which develop type III hyperlipidaemia, a chronic pro-inflammatory state that may confound the interpretation of results [243].

It has been shown that lipidation status has a profound impact on apoE function [398], which likely contributed to the diverse effects of recombinant, glia-lipidated and artificially lipidated apoE on MMP-9 conversion. This is consistent with previous studies demonstrating that various apoE preparations can yield dramatically different results [248], [383], [388], [398], [399]. The role and nature of apoE lipidation in health and disease are critical topics the field has been trying to reconcile for some time, which include the extent and composition of lipidation between apoE isoforms, changes during aging and disease, and differences between central and peripheral apoE, etc. As so little is known about the makeup of apoE particles under physiological conditions, the identification of appropriate lipidated preparations to study apoE and its function has proved to be challenging. The varied responses elicited by the different apoE preparations in the current studies highlight the need to identify physiologically relevant preparations of apoE, in order to more appropriately and reproducibly evaluate apoE function. Future *in vitro* experiments could utilise immortalised cell lines from primary glial cultures derived from apoE-TR mice for the production and purification of large quantities of apoE-containing lipoproteins [400]. Owing to the potential confounding influence of hyperlipidaemia in apoE2 homozygous mice, it may be necessary to instead use apoE2/3 heterozygous mice to avoid any lipid and cholesterol disruptions related to this condition.

In terms of MMP-9 activity, while an increased ratio of active:proMMP-9 was observed in the presence of the glia-lipidated apoE2 isoform, this did not translate to increased activity of MMP-

9. In fact, the presence of glia-lipidated apoE2 resulted in the largest inhibition of MMP-9 activity in a cell-free activity assay versus the other apoE isoforms. These supposedly contradicting results could be caused by the binding mechanism of each of the apoE isoforms. For instance, the different binding site or conformation adopted by the apoE2 isoform may enable the cleavage of MMP-9 by APMA however prevent MMP-9 from being able to bind and degrade substrates. MMP-9 can be inhibited by multiple mechanisms: binding of the inhibitor to proMMP-9 prevents conversion to the active form, whereas its binding to active MMP-9 allosterically inhibits MMP-9 activity. Lipidated apoE2 could conceivably be more suited to bind to active MMP-9, and subsequently inhibit its activity [401], [402].

I could not assess the binding affinities of each of the glia-lipidated apoE isoforms due to the presence of other proteins in the media which could interfere with the binding of apoE to MMP-9 or could bind to MMP-9 instead of apoE. To assess this in future studies, apoE would need to be isolated and purified before analysis. In the present studies, it was not possible to generate a sufficient amount of the necessary concentration of apoE from the primary glial cultures to perform these studies. Binding differences due to apoE lipidation state may arise from the conformational changes of apoE upon binding to lipids [403]–[406], which may also reveal underlying isoform-dependent differences. Although understanding the structure and the ensuing function of the apoE isoforms remains challenging due to the dynamic nature of their conformations, apoE4 is more likely to adopt a conformation that is considered pathological [407]. An important aspect of apoE4 structure is the orientation of Arg-61 which promotes domain interaction by interacting with Glu-255 within the lipid binding region, leading to a more compact conformation of apoE4 compared to the other isoforms [408], [409]. The binding affinities of each glia-lipidated apoE isoform to MMP-9 may follow a similar pattern to the delipidated, recombinant apoE (i.e. reduced binding with apoE4), as was reported in a study assessing the effect of lipidation status on the binding of apoE to TREM2, another risk factor for the development of AD; glia-lipidated apoE demonstrated similar binding affinities to delipidated recombinant apoE [116].

Alternatively, the effect of apoE lipidation status on the binding affinity to MMP-9 may be similar to that reported for the binding of apoE to Aβ, where lipidated apoE resulted in increased binding affinity and the emergence of apoE isoform differences [383].

Accordingly, the observed differences of the apoE isoforms on MMP-9 activity may be a result of the differences in the binding affinities of each apoE isoform to MMP-9, i.e., the greater the binding, the greater the inhibition of MMP-9 activity. This would suggest that apoE is directly inhibiting the enzymatic activity of MMP-9. ApoE is able to regulate the activity of enzymes involved in lipid metabolism [410] and APOE4 carriers have been previously shown to have altered global enzyme activity [341], [411], [412]. One mechanism by which apoE can alter the activities of enzymes is by binding to their receptors [347], [413], [414], while not much has been reported regarding the ability of apoE to inhibit enzymatic activity directly. Recently, apoE has been shown to bind and inhibit a key activator in the classical complement cascade, the C1q protein, thus inhibiting this inflammatory mechanism which plays a role in AD pathology [415], [416] and providing a clear example of a direct inhibitory role for apoE in AD.

The significant and dose-dependent inhibition of MMP-9 activity observed in the presence of each apoE isoform indicates an interaction between apoE and MMP-9. In addition to the binding studies, further evidence for this association was identified in a more physiological setting using high-resolution laser scanning microscopy. MMP-9 and apoE immunoreactivities clearly overlapped in numerous areas of the cortex of EFAD animals. The finding that apoE4 appeared to associate with MMP-9 to a lesser extent than apoE3 as a smaller fraction of MMP-9 was found to colocalise with apoE4 compared to apoE3, could be indicative of a weaker interaction between apoE4 and MMP-9. These studies are consistent with the kinetic binding studies above, which demonstrated a lower binding affinity of apoE4 to MMP-9, compared to other apoE isoforms. Collectively, these results suggest a physiologically relevant interaction between MMP-9 and apoE in the brain cortices of E3FAD and E4FAD mice.

Previously our group showed that inhibiting the proteolytic activity of MMP-9 reduces the lipoprotein receptors LRP1 and LDLR shedding and increases the clearance of Aβ through the BBB [182], [222]. As described in Chapter 2 and recently [319], MMP-9 expression and activity are elevated in AD cerebrovasculature relative to control subjects, which may have contributed to the reduced LRP1 expression observed in AD cerebrovasculature in the current studies. This data is consistent with prior reports showing LRP1 levels to be reduced in human AD brains [417], [418]. What has not been thoroughly investigated is the effect of APOE genotype on lipoprotein receptor levels in AD. Intriguingly, the present studies showed lipoprotein receptor levels were APOE genotype-dependent with the highest expression levels found in E4FAD mice and APOE4/4 individuals, most notably in the cerebrovasculature. Research conducted by Tachibana et al. (2019) demonstrated that LRP1 participates more in the clearance of Aβ in the context of the APOE3 genotype whereas with the APOE4 genotype, LRP1 tends to contribute more to Aβ seeding [418], [419]. Given that the seeding of Aβ into oligomeric and insoluble species, which accumulate in extracellular Aβ plaques, has been shown to accelerate Aβ pathology [420], [421], the elevated levels of LRP1 exhibited by individuals with the APOE4 genotype reported in this Chapter may ultimately be detrimental and contribute to AD pathology. ApoE4 has been shown to have a critical impact on the seeding of Aβ in the early stages of the disease before symptoms are apparent [422], [423], which may describe the increased LRP1 levels observed in the APOE4/4 non-demented controls.

While prior research from our group has shown that the APOE4 isoform is associated with increased shedding of LRP1 and LDLR *in vitro* and in apoE4-TR mice [182], the lower levels of LRP1 seen in the cerebrovasculature of human subjects did not translate to elevated levels of the soluble lipoprotein receptor in the brain. This is possibly due to soluble LRP1 binding to Aβ or other proteins and masking the epitope [177]–[181]. Similarly, no APOE isoform differences were apparent between E3FAD and E4FAD mice in the soluble brain fraction, however LRP1 levels did increase in E4FAD mice in 70-week-old relative to 40-week-old mice, which could be a result

of increased Aβ levels or inflammation in these mice promoting receptor shedding [177], [182], [424], [425].

Collectively, the results presented in Chapters 2 and 3 indicate that apoE influences MMP-9 disposition in the brain in an isoform-dependent manner, as displayed in Figure 3.9. ApoE was shown to influence both the levels of MMP-9 in conditioned media from brain endothelia and the conversion of proMMP-9 to active MMP-9, as well as dose-dependently inhibit MMP-9 activity. Importantly, overall, apoE4 was the least effective in modulating these processes compared to other apoE isoforms, which may be due to a weaker binding affinity and association with MMP-9. Both the human and animal AD brain specimens exhibited APOE isoform specific differences in MMP-9 levels, particularly in the cerebrovasculature. The elevated MMP-9 levels in APOE4 AD subjects may describe our prior work showing an effect of APOE genotype on lipoprotein receptor proteolysis and Aβ elimination across the BBB [122], [182], which are mediated by MMP-9 [222]. As such, targeting MMP-9 may be an effective strategy to mitigate AD pathophysiology, particularly for individuals with an APOE4 genotype.

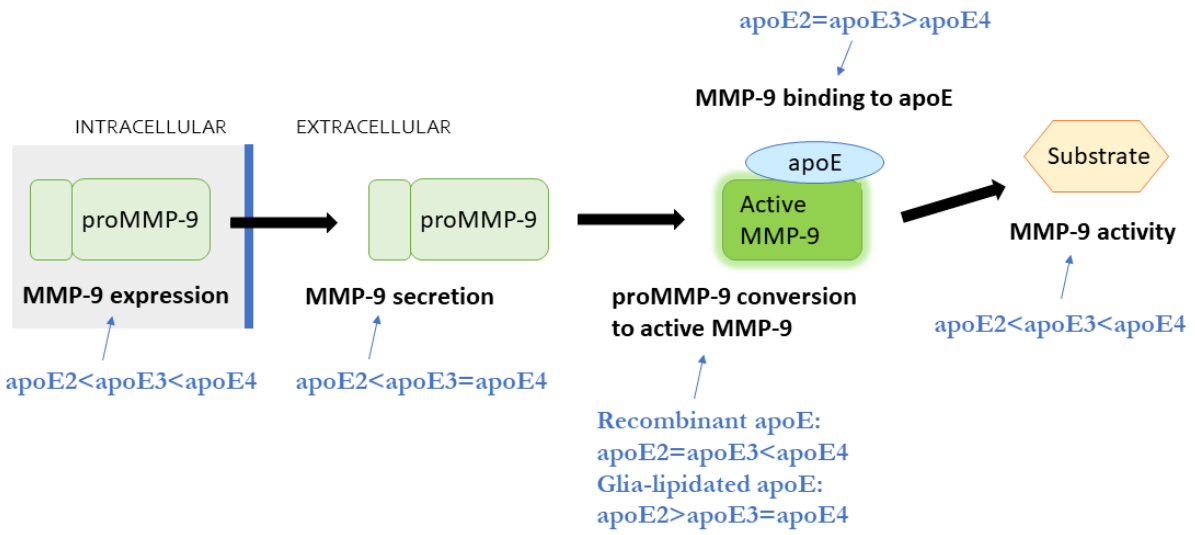

Figure 3.9 ApoE isoforms differentially regulate MMP-9 disposition.
A summary schematic depicting the results presented in Chapters 2 and 3, which demonstrate that apoE can influence multiple aspects of MMP-9 disposition in AD in an isoform-dependent fashion.

Chapter 4: Modulation of MMP-9 activity and expression in AD mice

4.1 Introduction

Elevated MMP-9 levels have been reported in numerous neurological and inflammatory disease states including AD [200], [208], [309], cerebral amyloid angiopathy [227], TBI [426], [427], amyotrophic lateral sclerosis [428], multiple sclerosis [429], [430], ischaemia [203], [204], intracerebral haemorrhage [431] and cardiovascular disease [193], [199]. Genetic knock out of the MMP-9 gene in mice has enabled the study of MMP-9 function in these pathological disease states. It has been demonstrated that MMP9KO mice were protected against cerebral ischemia [205] and TBI [206], reportedly due to the prevention of MMP-9 activity at the BBB. Pharmacologic inhibition of MMPs has been shown to attenuate tissue damage and oedema in cerebral focal ischemia [166], [432] and ameliorate neutrophil infiltration, oxidative stress, oedema and degenerating neurons in intracerebral haemorrhage [431]. In a transgenic mouse model of AD, minocycline treatment diminished inducible nitric oxide synthase and activation of microglia whilst ameliorating cognitive dysfunction which was attributed in part to the inhibition of MMP-9 [310]. MMP-9 has been found to play a causal role in Aβ-induced cognitive impairment and neurotoxicity [212]. While the injection of Aβ increases MMP-9 expression and induces hippocampal damage coupled with learning and memory deficits [212], [433]–[435], this was alleviated by MMP-9 inhibitors and diminished in MMP9KO mice [212].

Owing to the elevated MMP-9 levels in AD brains and the reduced ability of apoE4 to modulate MMP-9 disposition reported previously [319] and described in Chapters 2 and 3, the therapeutic value of reducing MMP-9 activity in E4FAD mice was investigated in this Chapter. For these studies, SB-3CT, which has been recognised as a selective MMP-2 and MMP-9 inhibitor [204], [436]–[438], was used to reduce MMP-9 activity. This compound readily crosses the BBB and was developed to circumvent the adverse effects associated with broad-spectrum MMP inhibitors [439], [440]. SB-3CT treatment for seven days has been reported to reduce neuronal laminin

degradation and protect neurons from ischemic cell death [436]. Furthermore, in a TBI model, SB-3CT treatment preserved hippocampal neurons from neurodegeneration and attenuated accompanying behavioural deficits [441]. This inhibitor has also demonstrated beneficial effects in animal models of stroke, subarachnoid haemorrhage and spinal cord injury [442]–[444]. While SB-3CT has shown efficacy in numerous neurological diseases, its effectiveness in AD has not been determined. A previous study investigated one aspect of AD pathology involving the shedding of pericyte NG2, a proteoglycan known to play a regulatory role in the maintenance of vascular integrity. While toxic oligomeric Aβ increased levels of activated MMP-9 which then enhanced the shedding of NG2 in primary human pericyte cultures, this was inhibited in the presence of SB-3CT [362].

Pharmacological inhibition of MMP-9 activity offers a potential approach for the treatment of AD, providing information about the timing and length of duration required for treatment. In addition to this approach, 5xFAD/MMP9KO mice were generated to investigate the effect of MMP-9 knockout from birth by crossing MMP9KO mice with 5xFAD mice. Many mouse models of AD, including 5xFAD mice, exhibit elevated levels of MMP-9 compared to WT mice, which may be contributing to the progression of AD pathology [445], [446]. Preventing this aberrant expression through the genetic manipulation of MMP-9 provides a more definitive approach to evaluating the role of MMP-9 in AD, separate from the influence of APOE isoforms. In this Chapter, the effects of MMP-9 inhibition and MMP-9 knockout on measures of behaviour (anxiety, sociability, social recognition memory and spatial memory), amyloid pathology and lipoprotein receptor shedding were assessed (Fig. 4.1).

4.2 Materials and methods

4.2.1 Materials

The MMP-9 inhibitor, SB-3CT, was purchased from Tocris Bioscience (Minneapolis, MN, USA). ELISA kits for mouse MMP-9 were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). ELISA kits for LRP1 and LDLR receptors were purchased from Cedarlane Labs (Burlington, NC, USA). Halt enzyme inhibitor cocktails, the BCA protein assay, HBSS and ELISA kits for human Aβ-40 and Aβ-42 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dextran and guanidine hydrochloride were purchased from MilliporeSigma (St. Louis, MO, USA).

4.2.2 Animals

4.2.2.1 5xFAD mice

5xFAD mice are APP/PS1 double transgenic mice that co-express five FAD mutations and additively increase Aβ-42 production resulting in a mouse model of AD with accelerated plaque development and elevated levels of cerebral Aβ-42 [236]. Of the three lines originally generated, the Tg6799 line expresses the highest levels of mutant APP and is the most widely used. The mice used in this study were on a congenic C57BL/6 background as opposed to the original hybrid B6SJL background and were hemizygous for the APP and PSEN1 transgenes. Extracellular amyloid plaques can be detected in the hippocampus, cortex and thalamus of these mice at 2 months of age and thioflavin-S-positive plaques appear between 2 and 4 months of age in the frontal, parietal and entorhinal cortices and the dentate gyrus [237], [238]. Mice exhibit progressive cerebral amyloid angiopathy from around 3 months of age [237]. Amyloid pathology is more severe in female compared to male mice [236], [239]. Spatial working memory is impaired starting from 3-6 months of age and is exacerbated with age. Anxiety decreases progressively with age from 3-6 months in this strain of mice as determined by the elevated plus maze [240].

4.2.2.2 EFAD mice

ApoE-TR mice were purchased from Taconic Biosciences (Rensselaer, NY) and allowed to adapt to the vivarium for 2 weeks prior to any experimental procedures. The apoE-TR mice were created by targeted replacement of the endogenous murine APOE gene with human APOE2, APOE3 or APOE4 [330]. These mice retain the endogenous regulatory sequences required for apoE production and express the human apoE protein at physiological levels. The EFAD animals were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). To generate the EFAD mouse model, 5xFAD mice (Tg6799 line) were crossed with apoE4, apoE3, and apoE2-TR mice, producing the E4FAD, E3FAD, and E2FAD mouse models respectively, as previously described [247]. The EFAD mice remain homozygous for the APOE allele and heterozygous for the 5xFAD mutations. Overall, the EFAD mice display a less severe AD phenotype compared to the 5xFAD line; they develop plaques in the subiculum and cortex at four months of age as opposed to two months in the 5xFAD line [247]. EFAD mice display age-dependent Aβ accumulation and deficits in cognitive function, both of which are APOE-specific; E4FAD mice demonstrate the greatest deficits in cognitive function and developed more Aβ pathology compared to E3FAD and E2FAD mice [247], [251]. Amyloid pathology is greater in female mice than male mice [250]. In this Chapter, inhibition of MMP-9 activity by treatment with SB-3CT was assessed in E4FAD mice.

4.2.2.3 MMP9KO mice

MMP9KO mice and their C57BL/6 controls were purchased from the Jackson Laboratory (JAX stock #007084, Bar Harbor, ME, USA) [447] and allowed to adapt to the vivarium for 2 weeks prior to any breeding or experimental procedures. MMP9KO mice were generated as previously described and are viable, fertile and shown to survive for at least 24 months [198]. No MMP-9 activity is detected in spleen cell lysates from these mice [198], a finding which was confirmed in the MMP9KO mice used in the current studies through the zymographic analysis of spleen cell lysates. Moreover, tail snips were assessed through Transnetyx, Inc. (Cordova, TN, USA) which

confirmed the absence of the MMP-9 gene. While long bones (tibia, femur) are initially 10% shorter in MMP9KO mice due to delayed apoptosis, vascularisation, and ossification, this is resolved by remodelling by 8 weeks of age [198].

4.2.2.4 Generation of the 5xFAD/MMP9KO mice

The genetic manipulation of MMP-9 levels was investigated by crossing MMP9KO mice with 5xFAD mice. Both strains of mice were on a C57BL/6 background. 5xFAD mice, hemizygous for the APP and PSEN1 transgenes were initially crossed with MMP9KO mice which were null for the MMP-9 gene. The resulting litter were all heterozygous for the MMP-9 gene, half were positive for the 5xFAD mutations (5xFAD/MMP9KO-het) and half were negative (WT/MMP9KO-het). The 5xFAD/MMP9KO-het mice were then backcrossed with the MMP9KO mice to generate 5xFAD/MMP9KO, WT/MMP9KO (MMP9KO), 5xFAD/MMP9KO-het and WT/MMP9KO-het mice. The presence or absence of genes was determined through the assessment of tail snips by Transnetyx, Inc. (Cordova, TN, USA). Concurrent breeding of 5xFAD and WT mice generated the cohort of 5xFAD and WT control mice used for the study. Gender matched, 6-month-old WT, 5xFAD, 5xFAD/MMP9KO, 5xFAD/MMP9KO-het and MMP9KO mice were used in this study (n=12, (6 x males, 6 x females). 5xFAD/MMP9KO-het mice were only used for pathological analysis.

4.2.2.5 Housing

Mice were housed under standard laboratory conditions (23 ± 1 °C, 50 ± 5% humidity, and a 12-hour light/dark cycle) with free access to food and water throughout the study. Mice were multi-housed through the elevated plus maze (EPM), open field test (OFT) and three-chamber tests. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

4.2.3 In vivo treatment with SB-3CT in E4FAD mice

SB-3CT has been shown to cross the BBB following intraperitoneal injection in mice [440]. In this study, 4-month-old E4FAD mice were injected intraperitoneally with either SB-3CT (25mg/kg) dissolved in 25% DMSO/65% Polyethylene glycol (PEG)-40/10% water or vehicle (25% DMSO/65% PEG-40/10% water alone) for 5 consecutive days per week for a total of 1 month. This duration was chosen to assess the effect of extended MMP-9 inhibition. Previously, seven days of treatment with this concentration of SB-3CT effectively inhibited MMP-9 in a mouse model of focal cerebral ischemia [436]. An intraperitoneal route of delivery was chosen for SB-3CT as the inhibitor has been shown to be effectively delivered to the brain via this route in previous studies [204], [436]. In total, 28 animals were treated, divided into two groups balanced for gender and weight (E4FAD-vehicle, n=14 (5 x males, 9 x females), E4FAD-SB-3CT, n=14 (5 x males, 9 x females)). Male and female mice weighed on average 32.41 ± 0.97 g and 21.57 ± 0.21 g, respectively. SB-3CT treatment was adjusted for weight (25mg/kg).

4.2.4 Study design

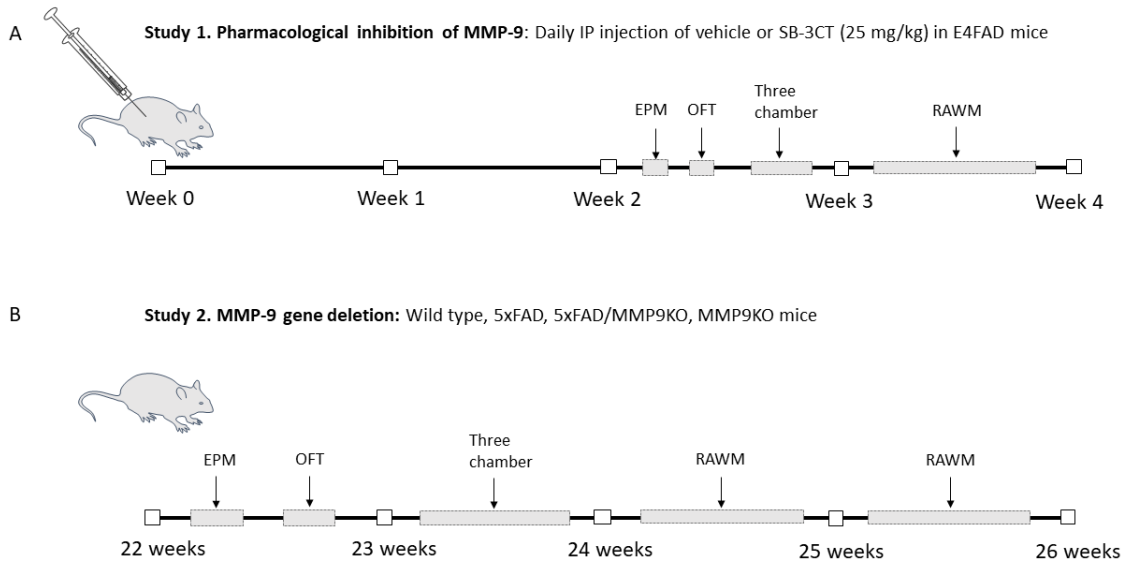

Figure 4.1: Study design for the 4-week pharmacological MMP-9 inhibition and MMP-9 gene deletion in vivo analysis.
(A) Study 1. MMP-9 was pharmacologically inhibited in E4FAD mice through a 4-week treatment with vehicle or SB-3CT (25 mg/kg), injected intraperitoneally (IP). (B) Study 2. MMP9KO mice were crossed with 5xFAD mice to create 5xFAD/MMP9KO mice, which were tested alongside WT, 5xFAD and MMP9KO mice. Behavioural analysis consisted of the EPM, OFT, three chamber test and the RAWM.

Daily intraperitoneal injections of vehicle or SB-3CT (25 mg/kg) were administered to 4-month-old E4FAD mice, for a period of 4 weeks (Fig. 4.1a). Behavioural analysis began following 2 weeks of injections and mice were euthanised after 4 weeks for pathological analysis. WT, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice were tested for behavioural changes at 22 weeks of age and euthanised aged 26 weeks along with 5xFAD/MMP9KO-het mice for pathological analysis (Fig. 4.1b).

4.2.5 Behavioural analysis

4.2.5.1 Evaluation of anxiety-related behaviour and motor activity in mice

Motor function and anxiety were assessed in E4FAD mice after 2 weeks of treatment of either SB-3CT (25mg/kg) or vehicle, and in WT, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice using the elevated plus maze and the open field test. The EPM consists of an elevated area (0.5m) with two open arms and two closed arms with 15 cm high walls and an open roof (similar arms are opposite each other) [448]. Mice were individually placed in the centre of the maze and movements were tracked using the EthoVision software for 5 minutes (Noldus, VA, USA). Mice were scored based on the number of entries into closed vs open arms and the time spent in closed vs open arms. An increase in open arm activity indicates anti-anxiety behaviour [449]. The OFT is a common measure of exploratory behaviour and general activity in mice [450]–[452]. The mice were individually placed into an enclosure with surrounding walls and an open roof and movements were tracked using the EthoVision software for 10 minutes (Noldus, VA, USA). Mice were scored based on the number of entries into the centre, middle and outer edges of the arena and the time spent in these three areas. An increase in duration/number of entries into the centre area indicates anti-anxiety behaviour [450]–[452]. The distanced travelled by the animal in the OFT provides a measure of motor activity [450], [451].

4.2.5.2 Assessment of social interaction and social memory in mice

The three-chamber test was used in these studies to measure cognition in the form of general sociability and interest in social novelty [453] in E4FAD mice following 2.5 weeks of SB-3CT treatment, and in WT, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice. Mice are typically social animals, preferring to spend more time with other mice, and have a natural tendency to investigate a novel mouse rather than a familiar one [454]. In this test, mice were placed individually into the centre chamber of a box arena with three equally sized chambers and openings between the chambers. A schematic of the setup is displayed in Figures 4.3 and 4.10. The two side chambers contained a wire cup through which the subject mouse can have indirect interaction with the novel and familiar mice. This test consisted of three 10-minute experimental sessions with different setups. In all sessions, the subject mouse could explore the whole arena. In the first session (habituation) the two side chambers remained empty to give the mice time to explore and become familiar with the arena. In the second session (test for social interaction), a mouse was added to the wire cup in one side chamber. In the third session (test for social memory), a novel mouse was placed in the wire cup in the opposite side chamber to the now familiar mouse. Positions of the novel and familiar mice were changed between trials to avoid side bias. The principle of the test is to present the subject mouse with a choice to spend time in any of the three compartments in the box arena during each of these experimental sessions and interact with mice that may be present [453]. Time spent in each chamber, time spent in the immediate area surrounding the wire cup, and the number of entries into each area were recorded. The test for social interaction measured the time spent with another mouse compared to time spent alone in an identical but empty chamber. The test for social memory measured the preference for a novel vs familiar mouse [453].

4.2.5.3 Assessment of spatial memory in mice

Spatial memory and learning was assessed in E4FAD mice after 3 weeks of treatment with either vehicle or SB-3CT, and in WT, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice using the radial

arm water maze (RAWM) [455]. The RAWM has been shown to be a sensitive measure for detecting learning ability and memory deficits in mice [456]–[458]. The mice underwent nine trials per day for a total of five consecutive days. The RAWM consists of a circular water-filled maze with six arms extending from an open central area that are distinguishable by unique visual cues on the end of each arm. A schematic of the setup is displayed in Figures 4.4 and 4.12. The mice were placed into one of the five entry arms (alternated between trials) and the task was to find the hidden platform in the sixth arm (goal arm), which remained constant. Each trial ended after one minute and errors were calculated after tracking the movements using the EthoVision software (Noldus, VA, USA). Entry into an incorrect arm was scored as an error. The total number of incorrect errors made per trial before finding the hidden platform reflects reference memory, while the number of incorrect re-entries (multiple entries into the same arm) indicates working memory. By the last day of trials, mice that have correctly learned the location of the hidden platform demonstrate errors of 1 or less and show improvement between trials [455]. Latency to reach the hidden platform was also recorded and analysed.

4.2.6 Isolation of brain fractions

Mouse brains were homogenised and the cerebrovasculature, parenchyma and soluble brain fraction were isolated using a step-wise density gradient extraction process as previously described [182]. Briefly, mouse brain samples were homogenised in cold HBSS using a Dounce homogeniser. The homogenates were suspended in HBSS with 20% dextran and centrifuged for 15 minutes at 6000g and 4 °C. The cerebrovascular pellet at the bottom of the tube was gently rinsed in HBSS and collected with lysis buffer (M-PER + 1% EDTA + 0.2% PMSF (Thermo Scientific, USA)) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). The remaining parenchyma and soluble brain fraction were centrifuged for a further 10 minutes to separate these two fractions. The parenchyma was resuspended in HBSS and centrifuged for a final 5 minutes before the pellet was collected in lysis buffer. All fractions were stored at -80 °C prior to analysis.

4.2.7 Guanidine extraction

Levels of soluble and insoluble Aβ40 and Aβ42 were analysed in the brain. Briefly, half of the parenchyma material was homogenised by sonication in lysis buffer (M-PER + 1% EDTA + 0.2% PMSF (Thermo Scientific, USA)) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA) on ice before centrifugation at 15000g for 30 minutes at 4 °C. The resulting supernatant was mixed with an equal amount of 5M guanidine in TRIS buffer generating the guanidine soluble (GS) fraction. For the guanidine insoluble (GI) fraction, the pellet was resolubilised in lysis buffer and combined with an equal amount of 5M guanidine solution. Both GS and GI fractions were subsequently incubated at room temperature for 1 hour and were mixed every 15 minutes. The GI fraction was centrifuged for another 30 minutes at 15000g, 4 °C to remove debris. All samples were stored at -80 °C prior to analysis. Quantification of Aβ40 and Aβ42 in the GS, GI, whole parenchyma, cerebrovascular and plasma fractions was carried out using an ELISA for human Aβ40 and Aβ42 (Invitrogen, USA).

4.2.8 Zymographic analysis of EFAD spleen samples

Spleen samples from SB-3CT and placebo-treated E4FAD mice were analysed for MMP-9 content through zymographic analysis as described in Chapter 2.2.7.

4.2.9 Statistical analysis

Data are expressed as mean ± SEM. Data was checked for normality and statistical significance was determined by ANOVA followed by the two-stage step-up method of Benjamini, Krieger and Yekutieli unless otherwise stated. A p-value lower than 0.05 was used to indicate a statistically significant difference. Statistical analyses were performed with GraphPad Prism 8.

4.3 Results

4.3.1 Pharmacological inhibition of MMP-9 activity with SB-3CT in E4FAD mice

4.3.1.1 SB-3CT treatment influenced anxiety levels but not motor activity in E4FAD mice

The EPM and the OFT revealed there were no differences in total distance travelled or average velocity between the SB-3CT-treated and vehicle-treated mice (Fig. 4.2b, c, e, f), indicating the drug treatment does not alter locomotor activity. Furthermore, while no effects in anxiety were demonstrated in the OFT (Fig. 4.2a), SB-3CT-treated mice spent significantly more time in the closed arm compared to control animals in the EPM (*$p<0.05$) (Fig. 4.2b). There were no gender differences observed in either the EPM or the OFT.

4.3.1.2 SB-3CT treatment did not impact social interaction, social memory, or spatial memory

E4FAD mice treated with either SB-3CT or vehicle showed no preference for the chamber or proximal zone containing a mouse compared to an empty cage in the three-chamber test (Fig. 4.3a, b). Furthermore, there were no differences between either group in the time spent with a novel mouse compared to a familiar mouse (Fig. 4.3c, d). There were no gender differences observed in the three-chamber test. In the RAWM, again no differences between the SB-3CT or vehicle-treated mice were observed in the number of incorrect entries made before finding the hidden platform (Fig. 4.4b), the latency to find the platform (Fig. 4.4c), the average distance travelled per trial (Fig. 4.4d) or the average velocity (Fig. 4.4e). Overall mice in both treatment groups continued to learn and improve each of the 5 days, making few incorrect entries and finding the hidden platform quickly. A schematic of the RAWM is shown in Figure 4.4a. When stratified for gender, male mice treated with SB-3CT displayed a slightly reduced latency to find the hidden platform, however this was not significant (Fig. 4.5c).

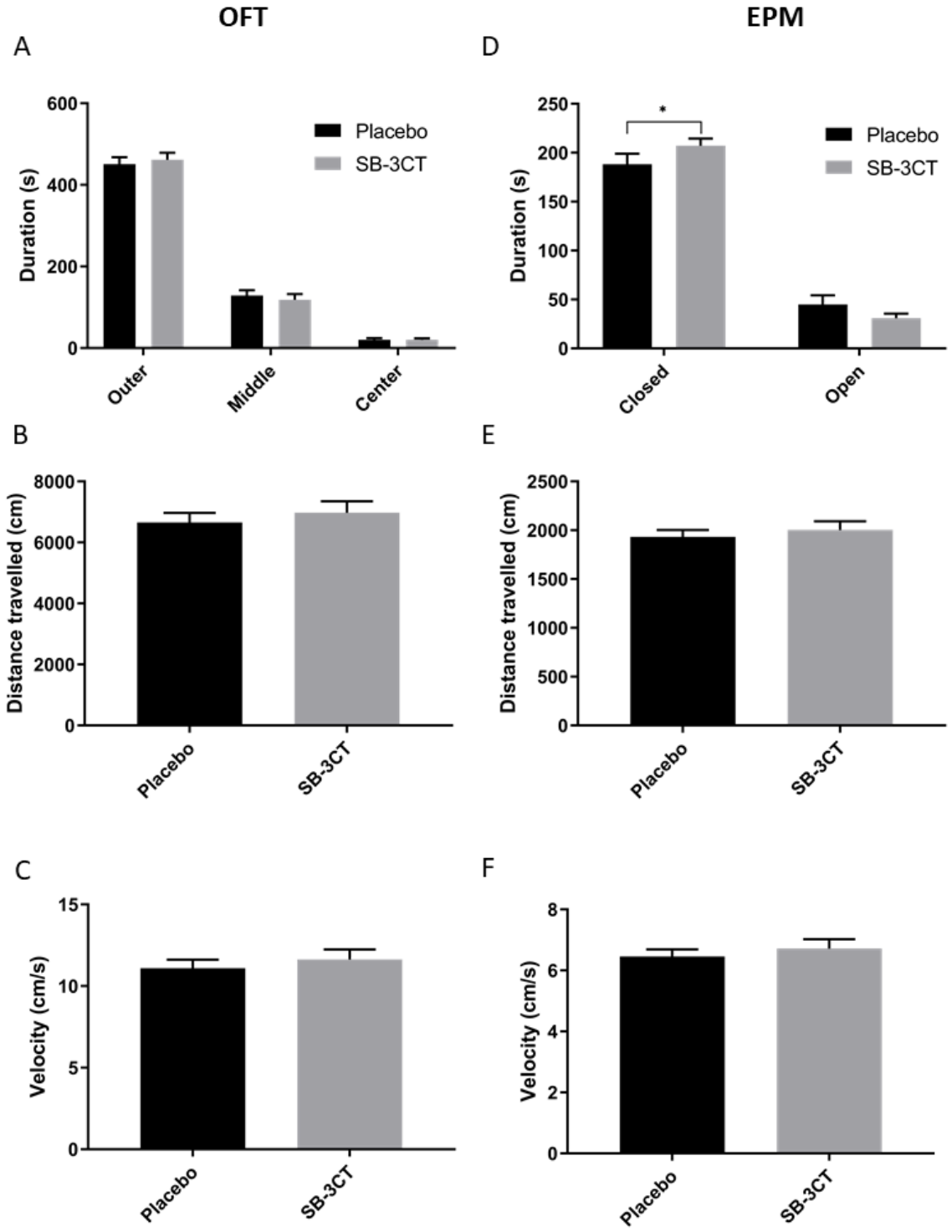

Figure 4.2: Anxiety-related behaviour and locomotor activity in the OFT and the EPM.
SB-3CT and vehicle-treated E4FAD mice were tested using (A, B, C) the OFT and (D, E, F), the EPM. (A) For the OFT, the duration spent in the outer, middle and centre areas of the circular arena was measured along with (B) the total distance travelled and (C) the average velocity. (D) For the EPM, the duration spent in the closed and open arms was measured together with (E) the total distance travelled and (F) the average velocity. Values represent mean ± SEM (N = 14). Statistical significance was determined by (A, D) two-way ANOVA followed by the BKY procedure and (B, C, E, F) an unpaired t-test. *$p<0.05$.

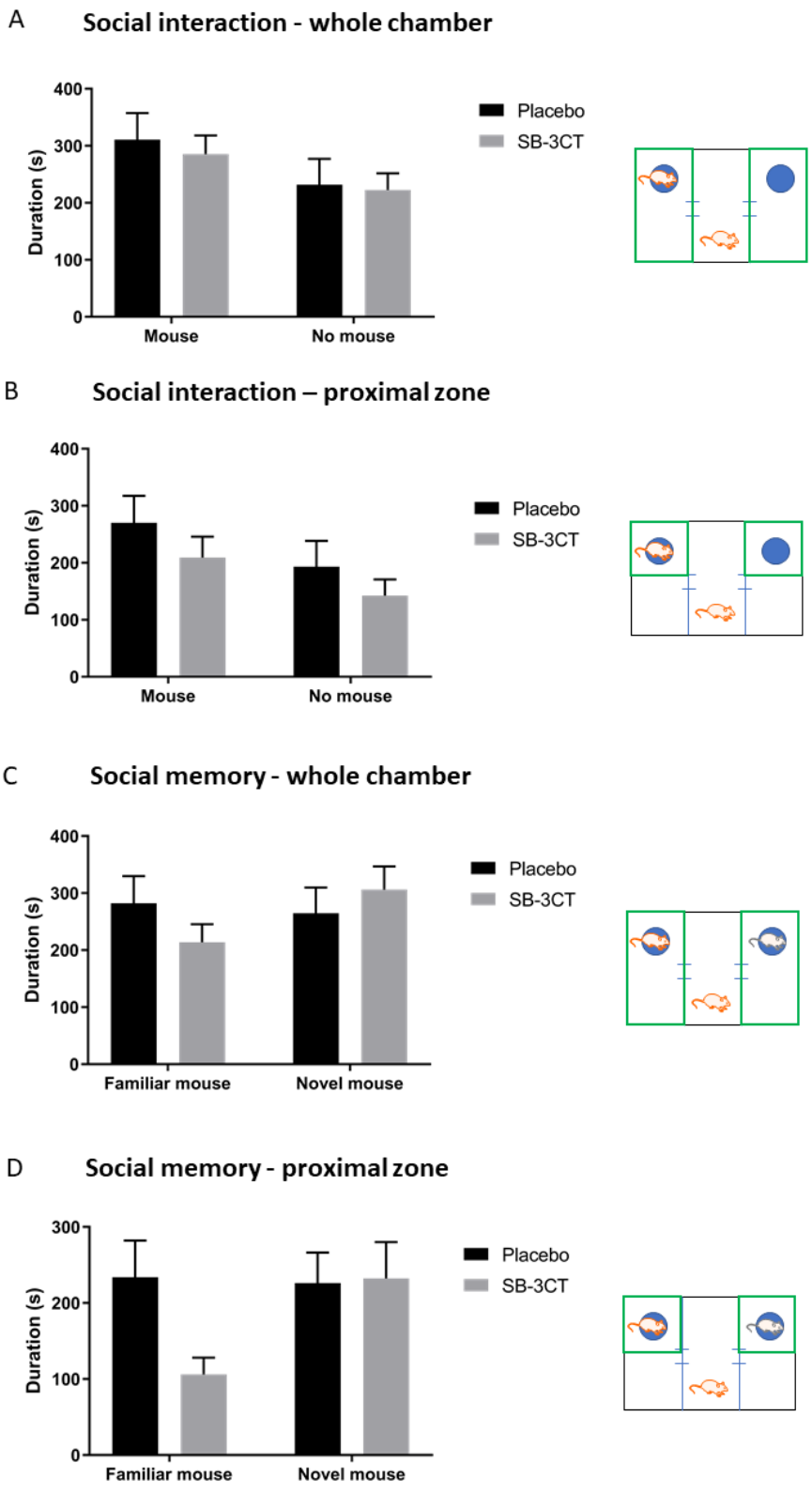

Figure 4.3: Testing social interaction and social memory using the three-chamber test.
SB-3CT and vehicle-treated E4FAD mice were tested for (A, B) social interaction (one mouse vs empty cage) and (C, D) social memory (novel mouse vs familiar mouse) in the three-chamber test. Time spent in (A, C) the whole chamber containing the mouse/empty cage and (B, D) the proximal zone surrounding the cages was measured (areas shown in green in each accompanying schematic). Values represent mean ± SEM (N = 14). No statistical significance was identified in any measures by a two-way ANOVA.

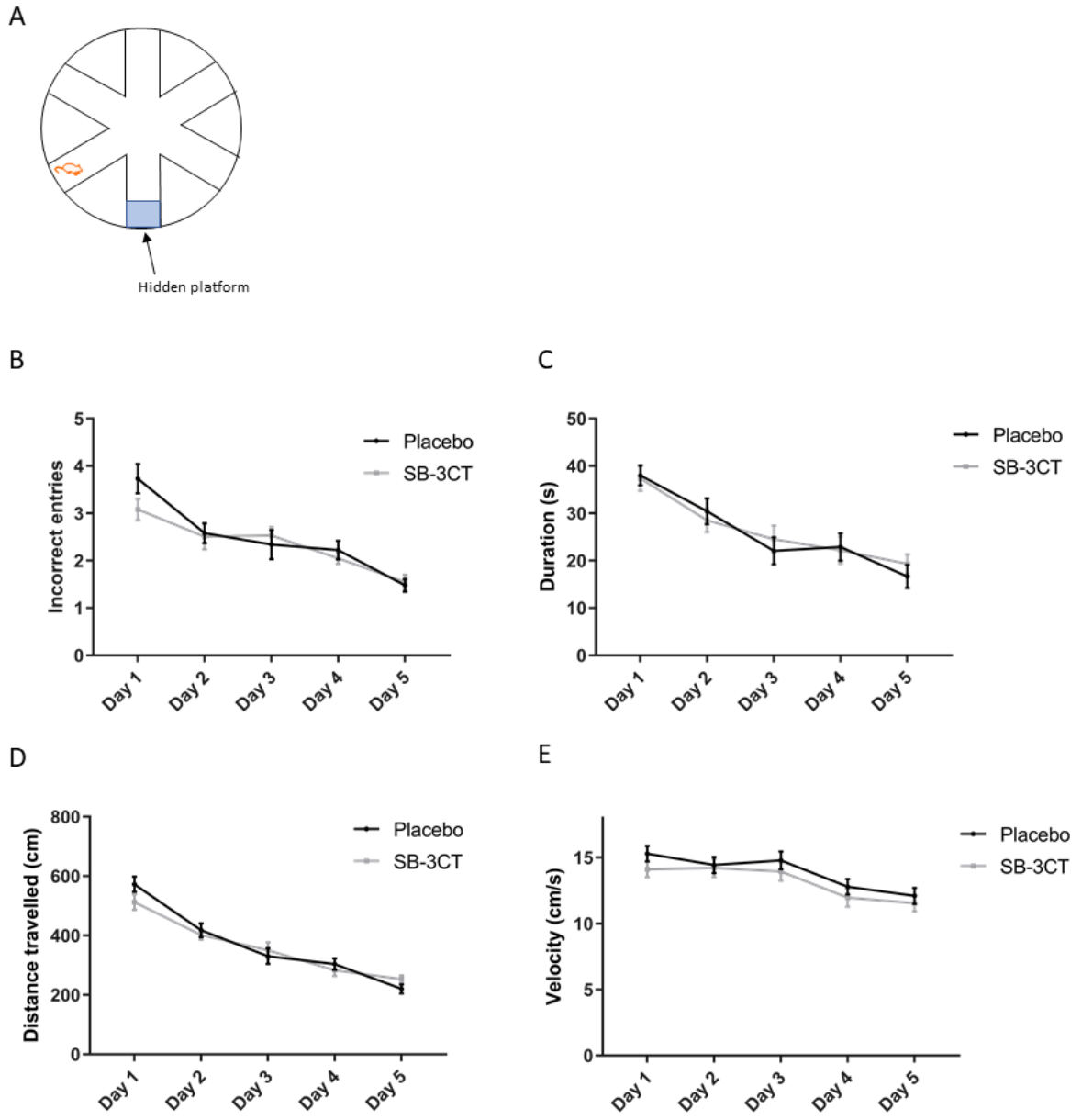

Figure 4.4: Spatial memory testing using the RAWM.
SB-3CT and vehicle-treated E4FAD mice were tested for their ability to find the hidden platform in the RAWM. (A) A schematic of the maze layout is displayed. Mice were tested in nine trials per day for 5 days. (B) The number of incorrect entries made and (C) the time taken to find the maze were recorded and analysed. (D) The total distance travelled per trial and (E) the average velocity while swimming was also evaluated. Values represent mean ± SEM (N = 14). No statistical significance was identified in any of the parameters by a two-way ANOVA.

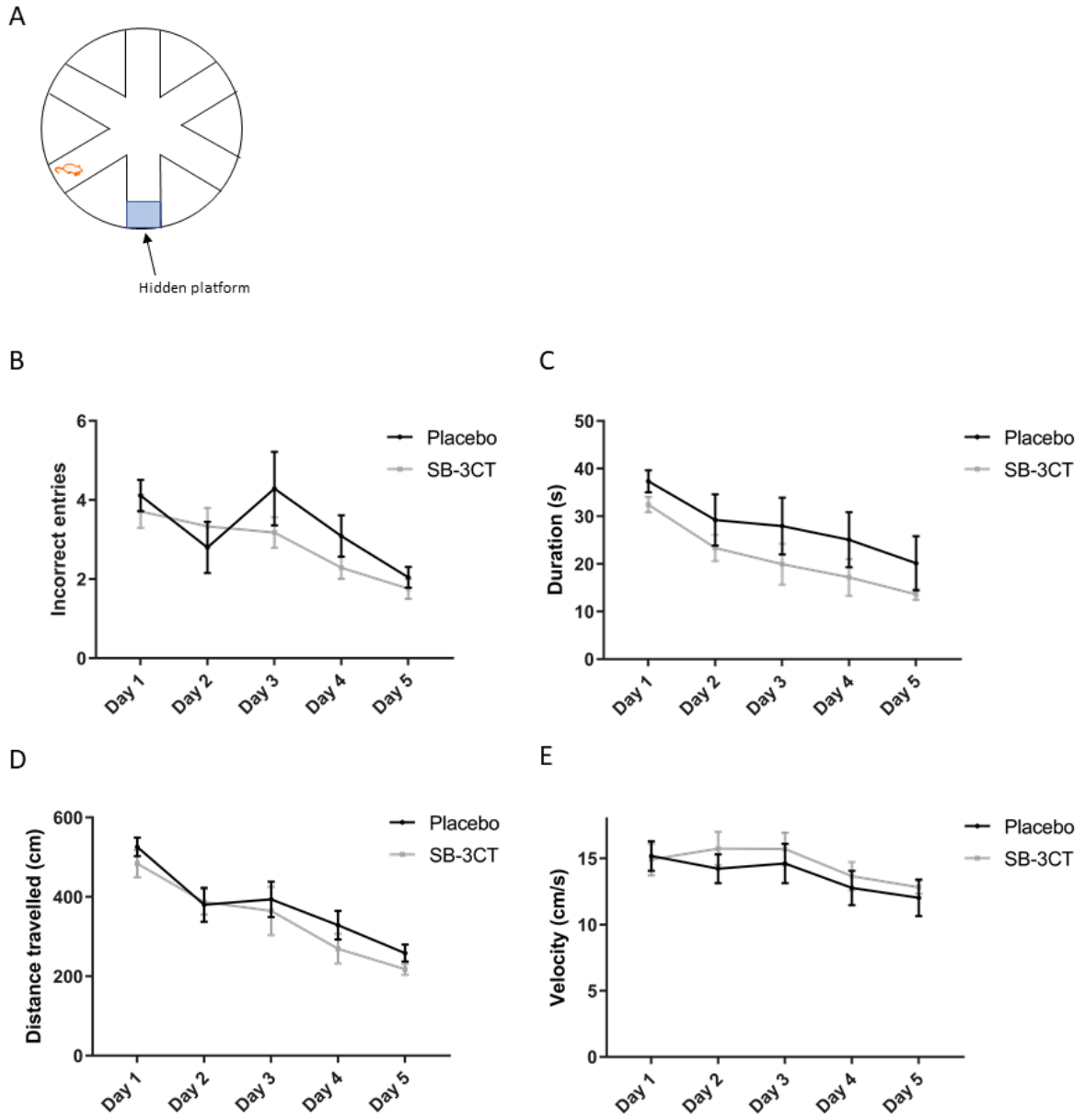

Figure 4.5: Spatial memory testing of male mice using the RAWM.
SB-3CT and vehicle-treated male E4FAD mice were tested for their ability to find the hidden platform in the RAWM. (A) A schematic of the maze layout is displayed. Mice were tested in nine trials per day for 5 days. (B) The number of incorrect entries made and (C) the time taken to find the maze were recorded and analysed. (D) The total distance travelled per trial and (E) the average velocity while swimming was also evaluated. Values represent mean ± SEM (N = 5). No statistical significance was identified in any of the parameters by a two-way ANOVA.

4.3.1.3 Amyloid levels remained similar for SB-3CT and vehicle-treated E4FAD mice

Aβ-40 and Aβ-42 levels were evaluated in the brain parenchyma fractions of SB-3CT and vehicle-treated E4FAD mice. The whole parenchyma brain fraction was analysed in addition to the guanidine soluble and insoluble parenchyma fractions. In addition, Aβ-42 levels were analysed in the cerebrovasculature of each mouse. While no differences in Aβ-40 and Aβ-42 levels were detected in any fraction, amyloid levels were typically higher in female mice compared to male mice (*$p<0.05$, **$p<0.01$, ***$p<0.001$) (Fig. 4.6).

4.3.1.4 LDLR and LRP1 levels were unchanged across treatment groups in E4FAD mice

LDLR and LRP1 were measured in the cerebrovasculature and soluble brain fraction of SB-3CT and vehicle-treated E4FAD mice (Fig. 4.7). Levels of both receptors remained unchanged in both brain fractions of SB-3CT and vehicle-treated mice (Fig. 4.7a, b, c, d). There were no gender differences observed in the analysis of LRP1 or LDLR.

4.3.1.5 Pro and total MMP-9 levels were unaltered by SB-3CT treatment

To examine the effects of the MMP-9 inhibitor (SB-3CT) on MMP-9 activity, the spleens of each mouse were examined via zymography (Fig. 4.8a, b), owing to the high expression levels of MMP-9 in this tissue type [459]–[461]. Of note, neither pro nor active MMP-9 levels were high enough in the mouse brain tissue samples to be detected via zymographic analysis. ProMMP-9 levels were detected in the spleens of the mice; however, no differences were identified between mice treated with SB-3CT or vehicle (Fig. 4.8a, b). Furthermore, to investigate whether total MMP-9 expression levels in the brain were altered by SB-3CT treatment, cerebrovasculature and parenchyma brain samples were analysed for MMP-9 in an ELISA. No differences in total MMP-9 levels were identified between SB-3CT and vehicle-treated mice in either fraction (Fig. 4.8c, d).

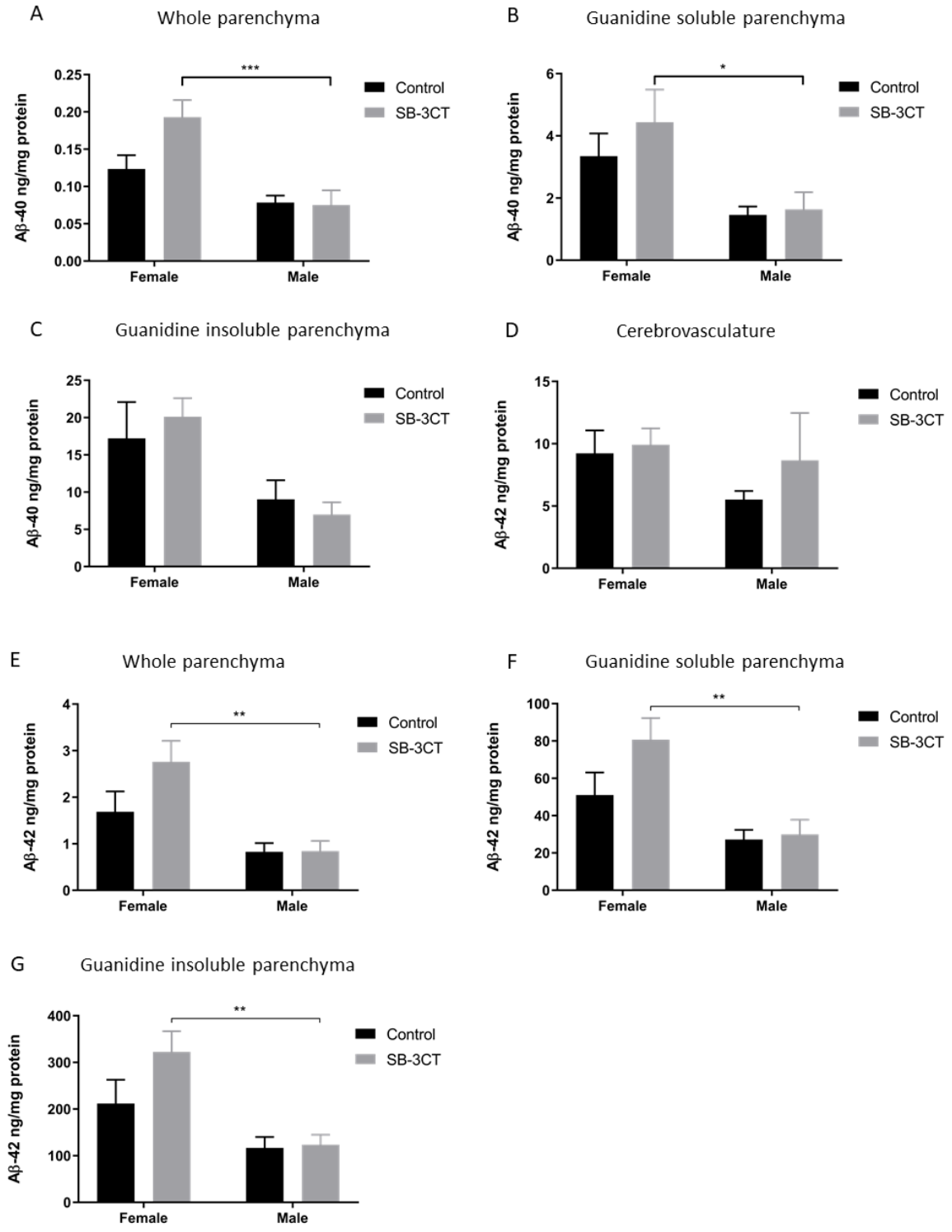

Figure 4.6: Analysis of Aβ-40 and Aβ-42 levels in the cerebrovasculature, whole parenchyma and the GS and GI parenchyma brain fractions.

Aβ-40 levels were examined in the (A) whole parenchyma, (B) GS parenchyma and (C) GI parenchyma of E4FAD mice. Aβ-42 levels were examined in the (D) cerebrovasculature, (E) whole parenchyma, (F) GS parenchyma and (G) GI parenchyma of E4FAD mice. Males and females were analysed separately due to large differences in amyloid levels. Values represent mean ± SEM (females: N = 9 per group, males: N= 5 per group). Statistical significance was determined by two-way ANOVA followed by the BKY procedure. *p<0.05, **p<0.01 ***p<0.001.

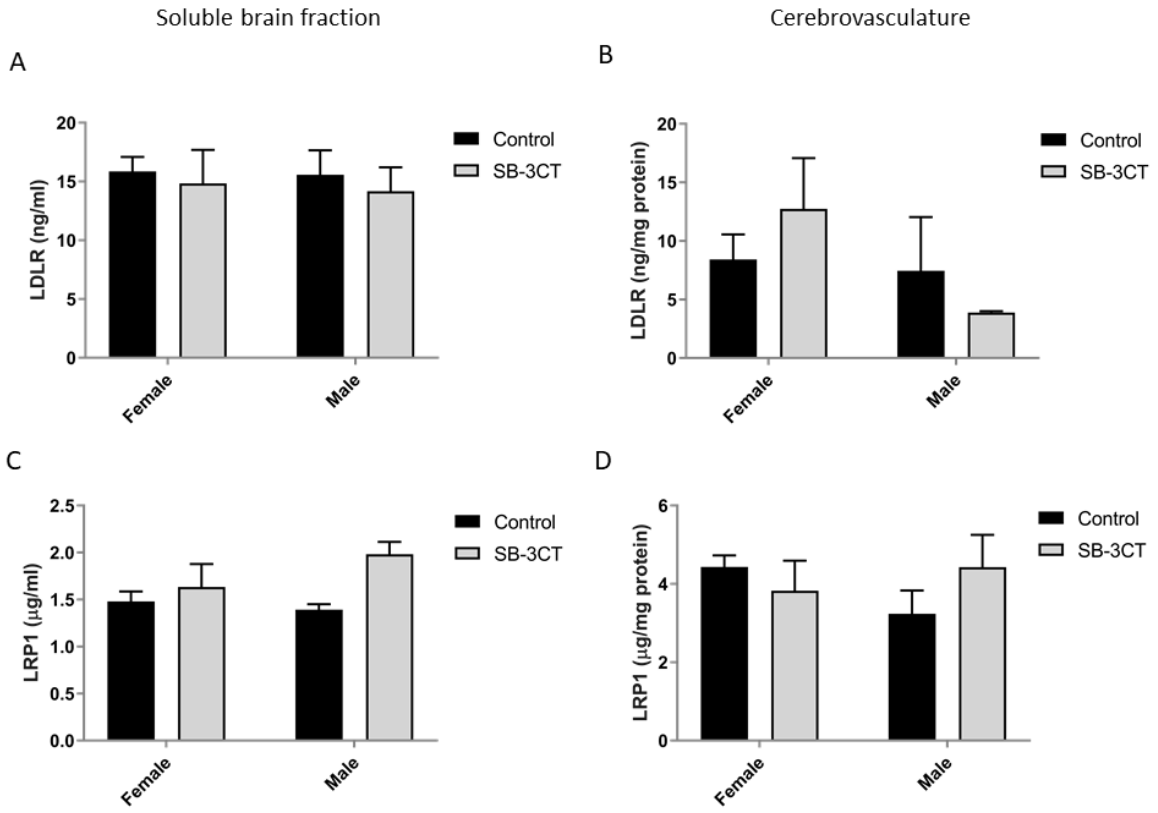

Figure 4.7: Analysis of LDLR and LRP1 levels in the cerebrovasculature and the soluble brain fraction.

Levels of the (A, B) LDLR receptor and (C, D) LRP1 receptor were analysed in the (A, C) soluble brain fraction and (B, D) the cerebrovasculature of SB-3CT and vehicle-treated E4FAD mice. Values represent mean ± SEM (females: N = 9 per group, males: N= 5 per group). No statistically significant differences in LDLR or LRP1 levels were identified between vehicle or SB-3CT-treated mice in either brain fraction by a two-way ANOVA.

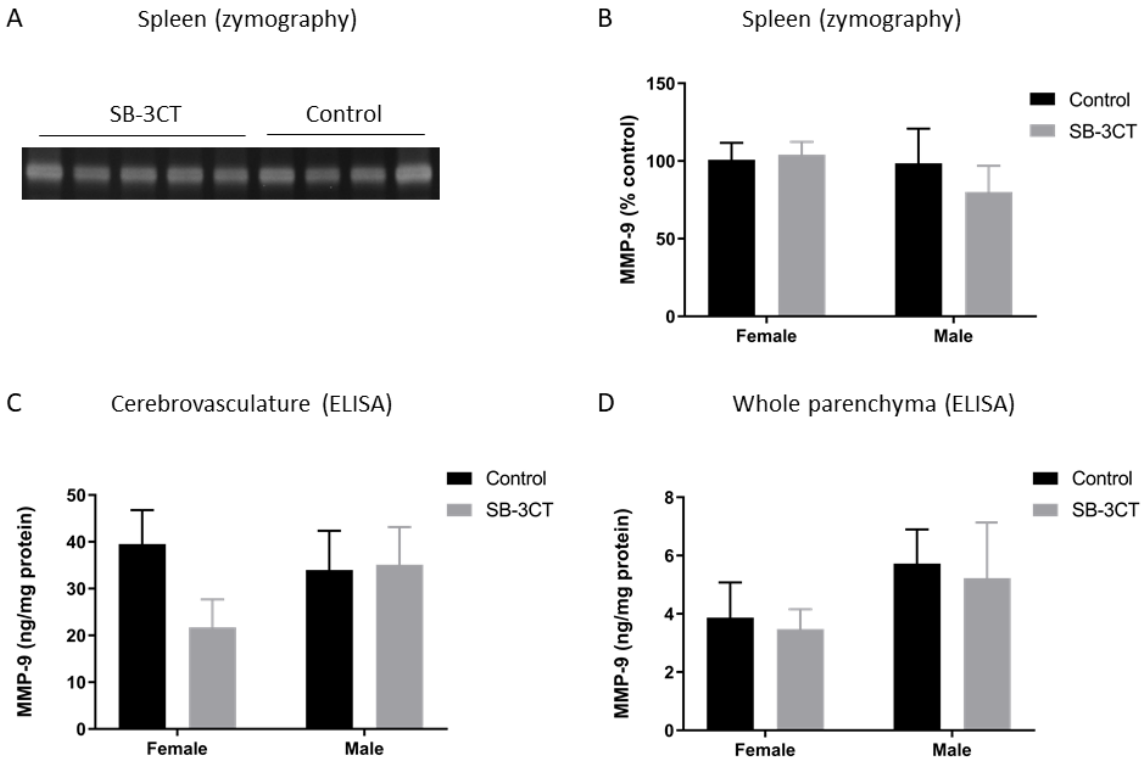

Figure 4.8: Analysis of MMP-9 levels in SB-3CT and vehicle-treated E4FAD mice.
(A, B) Levels of proMMP-9 were examined in spleen samples from SB-3CT and vehicle-treated mice by zymography. (A) Zymography gel showing bands of proMMP-9 in SB-3CT-treated and control animals. (B) Quantification of zymographic analysis. (C, D) Levels of total MMP-9 as measured by ELISA analysis of the (C) cerebrovasculature and (D) the whole brain parenchyma of SB-3CT and vehicle-treated E4FAD mice. Values represent mean ± SEM (females: N = 9 per group, males: N= 5 per group). No statistical significance was identified in any fraction by a two-way ANOVA.

4.3.2 Genetic manipulation of MMP-9 in 5xFAD mice

4.3.2.1 Anxiety and motor function differ between 5xFAD and 5xFAD/MMP9KO mice

The OFT demonstrated that 5xFAD mice exhibit reduced anxiety compared to all other genotypes (Fig. 4.9a). WT, 5xFAD/MMP9KO and MMP9KO mice spent more time close to the walls in the outer area of the arena, while 5xFAD mice spent significantly less time in this area (**$p<0.01$). Instead, 5xFAD mice spent significantly more time in the middle area of the arena, away from the walls, than the other genotypes (*$p<0.05$) (Fig. 4.9a). Results showing the time spent in the closed and open arms of the EPM indicate that 5xFAD mice spent less time in the closed arms compared to MMP9KO mice (*$p<0.05$) (Fig. 4.9d). In the OFT, the total distance travelled, and the average velocity were both reduced in 5xFAD/MMP9KO compared to 5xFAD and WT mice (*$p<0.05$, **$p<0.01$) (Fig. 4.9b, c). In the EPM, these parameters were both increased in 5xFAD mice compared to 5xFAD/MMP9KO and MMP9KO mice (*$p<0.05$) (Fig. 4.9e, f). There were no gender differences observed in either the EPM or the OFT.

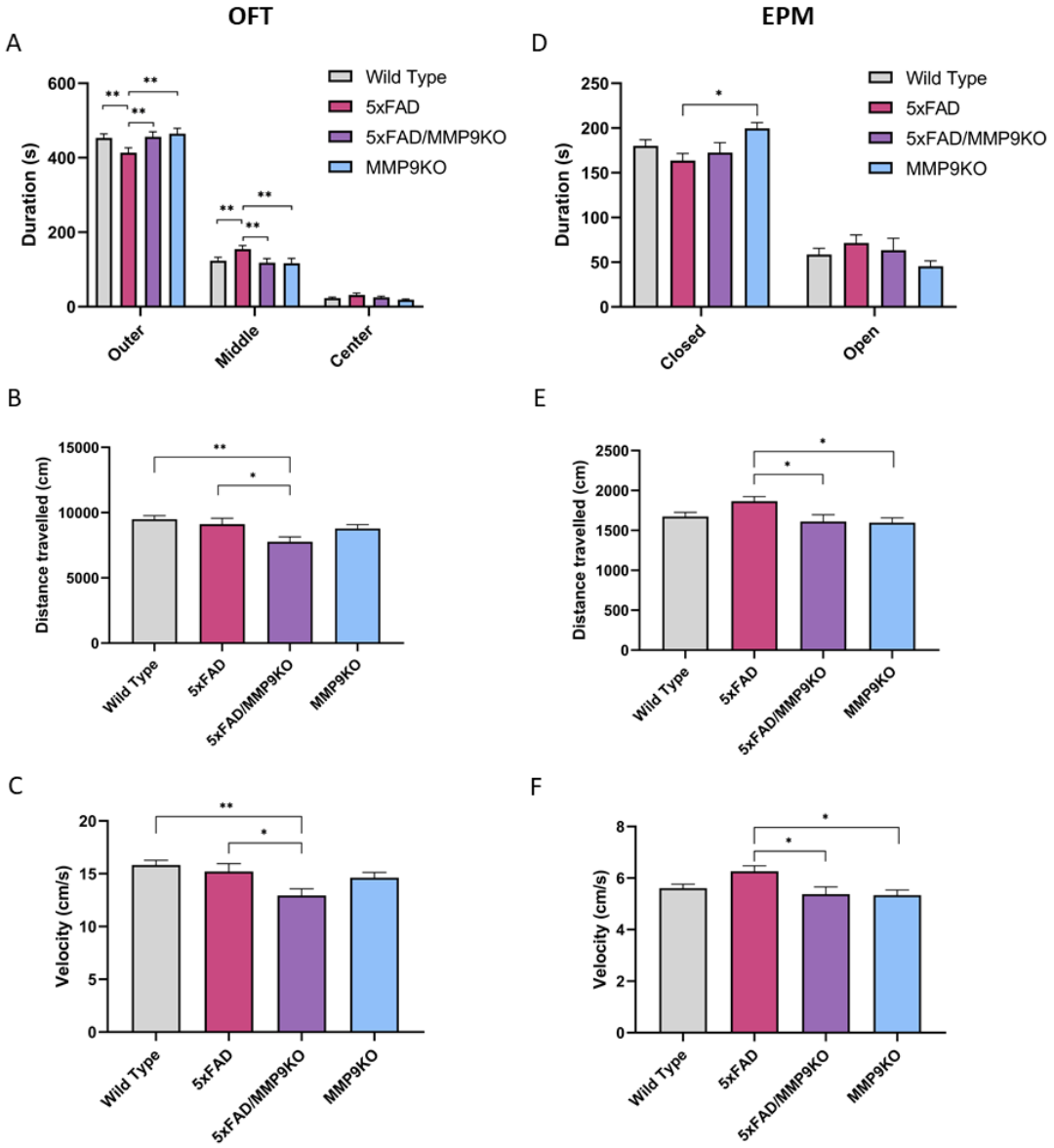

Figure 4.9: Anxiety-related behaviour and locomotor activity in the OFT and the EPM.
Wild type, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice were tested using (A, B, C) the OFT and (D, E, F), the EPM. (A) For the OFT, the duration spent in the outer, middle, and centre areas of the circular arena was measured along with (B) the total distance travelled and (C) the average velocity. (D) For the EPM, the duration spent in the closed and open arms was measured together with (E) the total distance travelled and (F) the average velocity. Values represent mean ± SEM (N = 12). Statistical significance was determined by (A, D) two-way ANOVA and (B, C, E, F) one-way ANOVA followed by the BKY procedure. *p<0.05, **p<0.01.

4.3.2.2 Genetic deletion of the MMP-9 gene rescues social memory deficits in 5xFAD mice

The EPM and OFT were followed by the three-chamber test to assess social interaction behaviour (general sociability) and interest in social novelty in each genotype group. Regarding sociability, all genotypes spent significantly more time in the chamber with another mouse compared to the chamber with an empty cage (*$p<0.05$, **$p<0.01$, ***$p<0.001$) (Fig. 4.10a). When examining the time spent in close proximity to the empty cage or the cage containing a mouse, again, all genotypes spent more time in the proximal zone surrounding the cage containing the mouse compared to the empty cage (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$) (Fig. 4.10b). However, 5xFAD mice spent significantly less time interacting with the mouse compared to WT mice ($p<0.05$) (Fig. 4.10b). Following the introduction of a novel mouse in the test for social memory, WT, 5xFAD/MMP9KO and MMP9KO mice all spent significantly more time with the novel mouse compared to the familiar mouse. Conversely, 5xFAD mice did not show a significant preference for either the novel or the familiar mouse (Fig. 4.10c, d). Overall, 5xFAD mice showed a lack of social interaction and increased deficits in social memory as demonstrated by a greater disinterest in exploring the novel mouse compared to the empty cage, and the novel mouse compared to the familiar mouse, respectively. Notably, this was not observed in the 5xFAD/MMP9KO mice, which exhibited behaviour akin to the WT and MMP9KO mice (Fig. 4.10c, d). Further analysis of this data revealed that these observed differences were driven by the male mice in each group (Fig. 4.11). When analysing the males alone, the previously observed differences were more pronounced and additional differences were identified. For example, when examining the proximal zone near the cages containing the mice, male 5xFAD/MMP9KO mice spent significantly more time with the novel mouse compared to the familiar mouse (*$p<0.05$) (Fig. 4.11d).

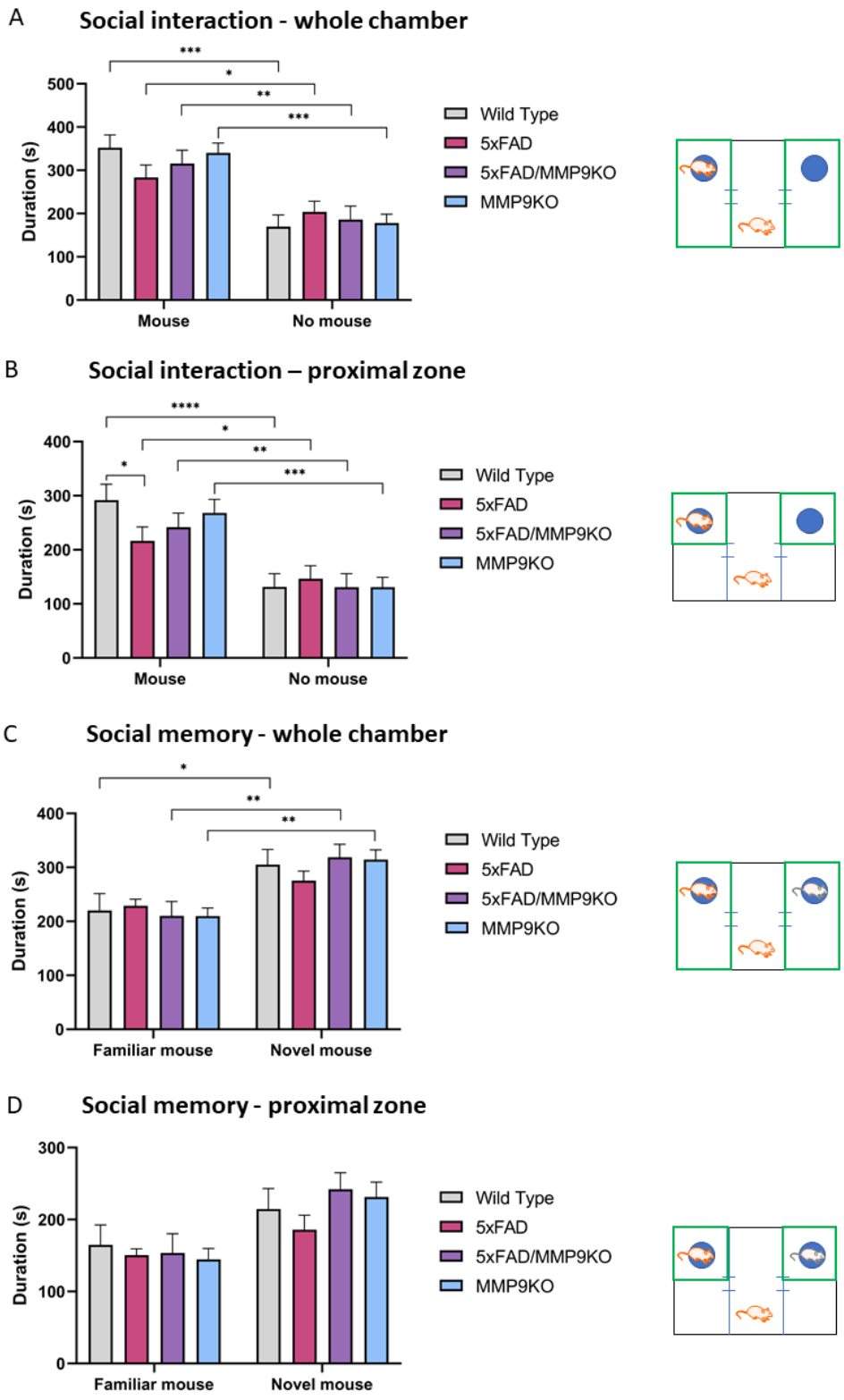

Figure 4.10: Testing social interaction and social memory using the three-chamber test.
Wild type, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice were tested for (A, B) social interaction (one mouse vs empty cage) and (C, D) social memory (novel mouse vs familiar mouse) in the three-chamber test. Time spent in (A, C) the whole chamber containing the mouse/empty cage and (B, D) the proximal zone surrounding the cages was measured (areas shown in green in each accompanying schematic). Values represent mean ± SEM (N = 12). Statistical significance was determined by a two-way ANOVA followed by the BKY procedure. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

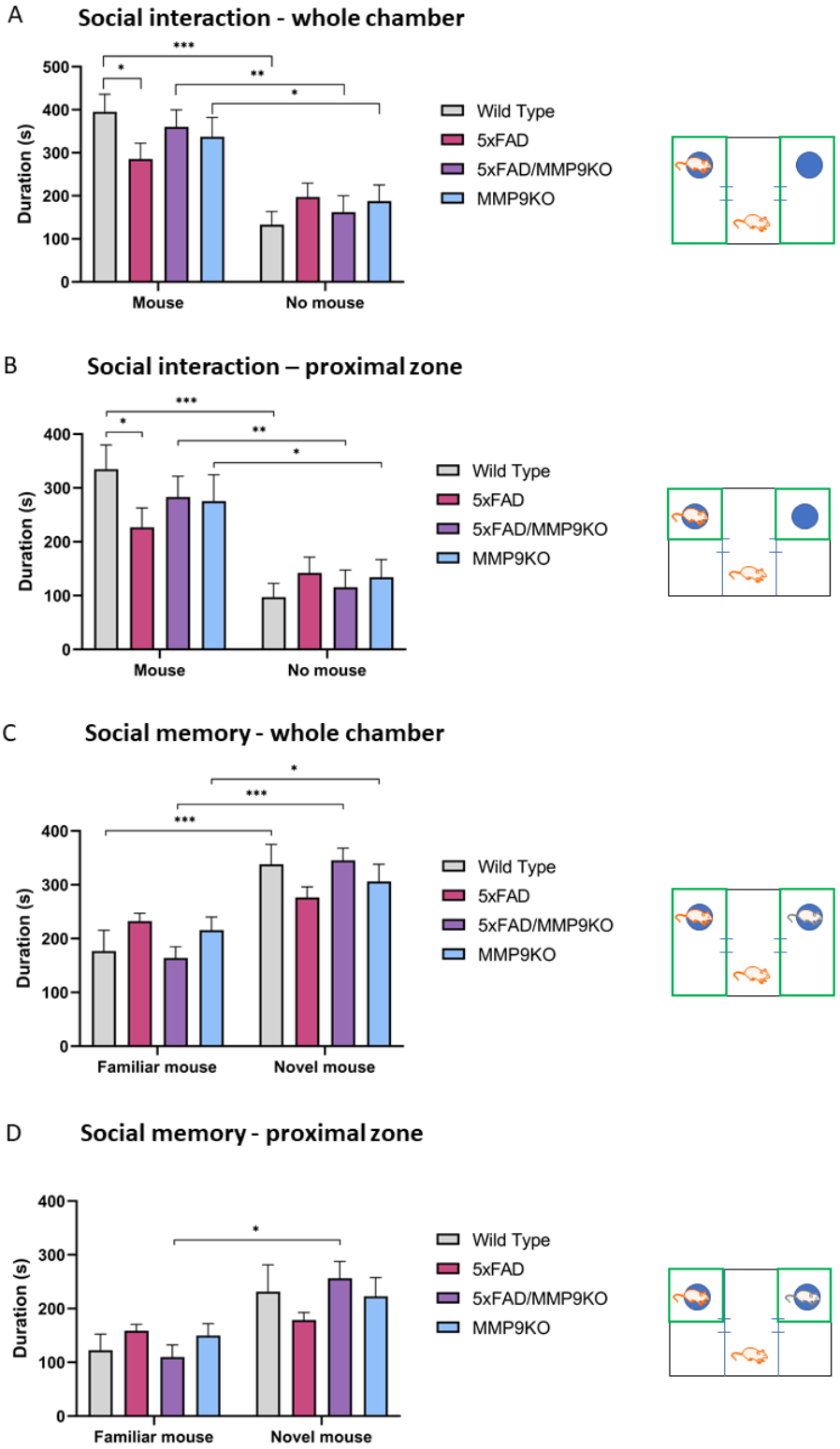

Figure 4.11: Testing social interaction and social memory of male mice using the three-chamber test.

Male wild type, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice were tested for (A, B) social interaction (one mouse vs empty cage) and (C, D) social memory (novel mouse vs familiar mouse) in the three-chamber test. Time spent in (A, C) the whole chamber containing the mouse/empty cage and (B, D) the proximal zone surrounding the cages was measured (areas shown in green in each accompanying schematic). Values represent mean ± SEM (N = 6). Statistical significance was determined by a two-way ANOVA followed by the BKY procedure. *p<0.05, **p<0.01, ***p<0.001.

4.3.2.3 No deficits in spatial memory were identified in 5xFAD or 5xFAD/MMP9KO mice

The RAWM was used to investigate working and reference memory (Fig. 4.12a). Analysis of the WT, 5xFAD, 5xFAD/MMP9KO or MMP9KO mice in the RAWM revealed no differences in the number of incorrect entries made for any genotype (Fig. 4.12b). All genotypes made gradually fewer mistakes as the trials progressed and the time taken to reach the platform also decreased by day 5 of testing. Again, no differences between genotypes were identified (Fig. 4.12c). The number of incorrect re-entries (multiple entries into the same arm per trial) was also assessed however, after day 1, this type of error was no longer made. When examining the distance travelled and the average velocity, no differences were detected between any groups (Fig. 4.12d, e). Overall, this data indicates that all groups showed continued improvement over the 5 days of trials, making few mistakes, and finding the platform quickly, even the 5xFAD mice. There were no gender differences observed in the RAWM.

4.3.2.4 Amyloid levels were similar across genotypes

Aβ-40 and Aβ-42 levels were evaluated in the cerebrovasculature and the brain parenchyma fractions of 5xFAD, 5xFAD/MMP9KO and 5xFAD/MMP9KO-het mice (Fig. 4.13, 4.14). WT and MMP9KO mice were excluded from analysis due to negligible Aβ levels. The parenchyma brain fraction was also analysed for soluble and insoluble amyloid following extraction with guanidine hydrochloride. In addition, Aβ-40 levels were analysed in the plasma of each mouse (Fig. 4.13). While no differences in Aβ-40 and Aβ-42 levels were detected in any fraction, amyloid levels were typically higher in female mice compared to male mice (*$p<0.05$, **$p<0.01$, ***$p<0.001$) (Fig. 4.13, 4.14).

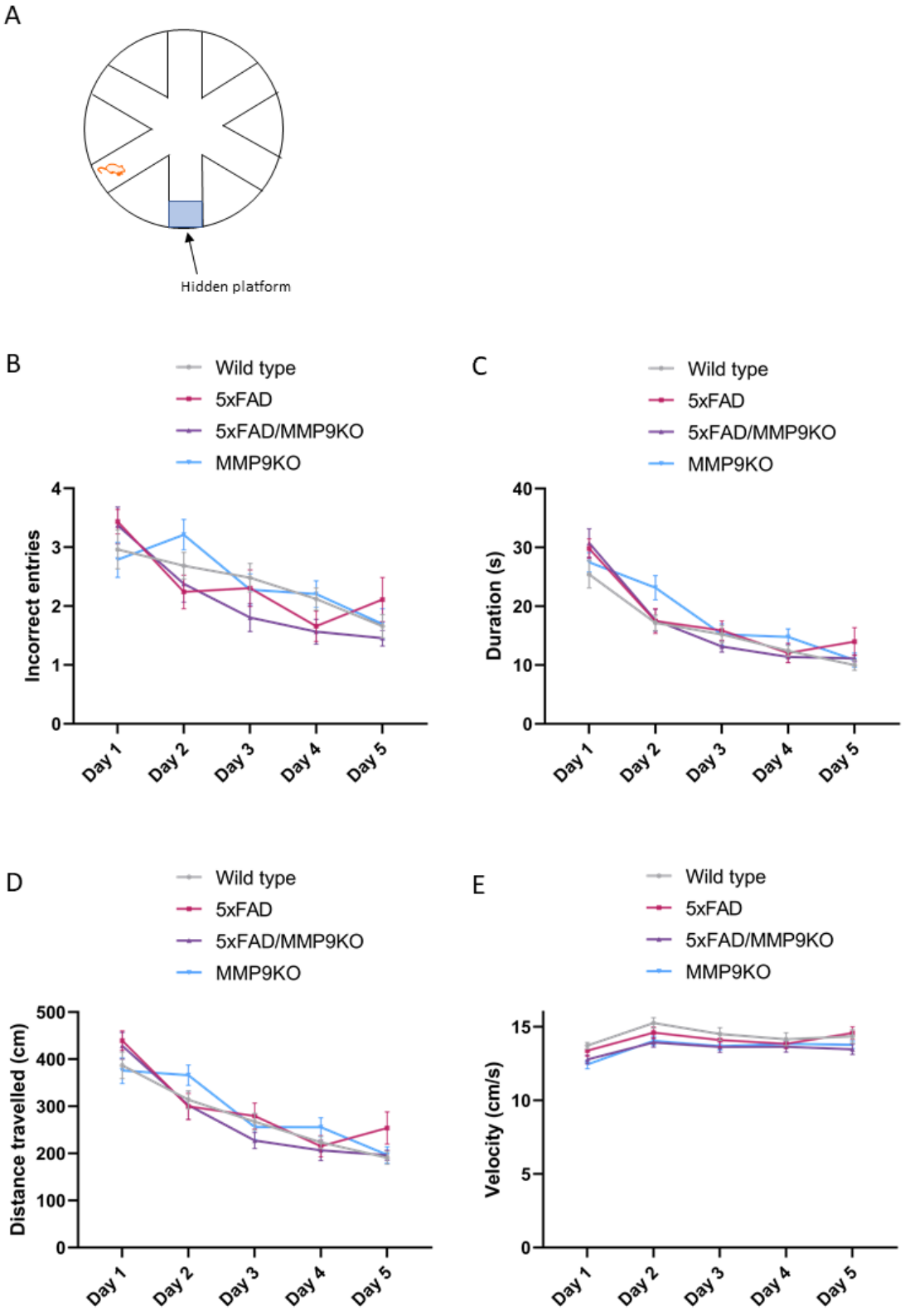

Figure 4.12: Testing spatial memory using the RAWM.
Wild type, 5xFAD, 5xFAD/MMP9KO and MMP9KO mice were tested for their ability to find the hidden platform in the RAWM. (A) A schematic of the maze layout is displayed. Mice were tested in nine trials per day for 5 days. (B) The number of incorrect entries made and (C) the time taken to find the maze were recorded and analysed. (D) The total distance travelled per trial and (E) the average velocity while swimming were also evaluated. Values represent mean ± SEM (N = 12). No statistical significance was identified in any of the parameters by a two-way ANOVA.

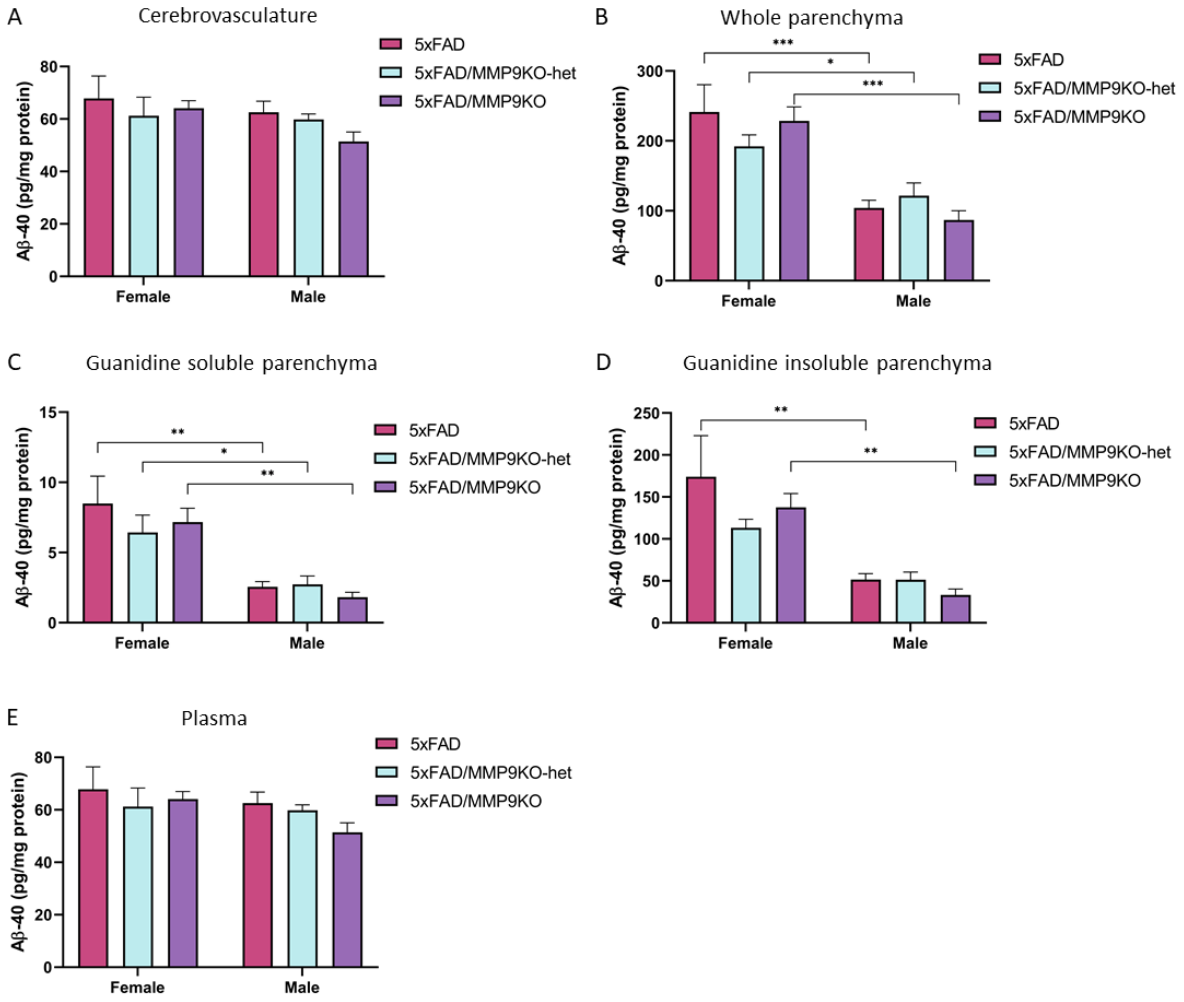

Figure 4.13: Analysis of Aβ-40 levels in the cerebrovasculature, plasma, whole parenchyma and the GS and GI parenchyma brain fractions.
Aβ-40 levels were examined in the (A) cerebrovasculature, (B) whole parenchyma, (C) GS parenchyma, (D) GI parenchyma and (E) plasma of 5xFAD, 5xFAD/MMP9KO and 5xFAD/MMP9KO-het mice. Males and females were analysed separately due to large differences in amyloid levels. Values represent mean ± SEM (N = 6). Statistical significance was determined by two-way ANOVA followed by the BKY procedure. *p<0.05, **p<0.01, ***p<0.001.

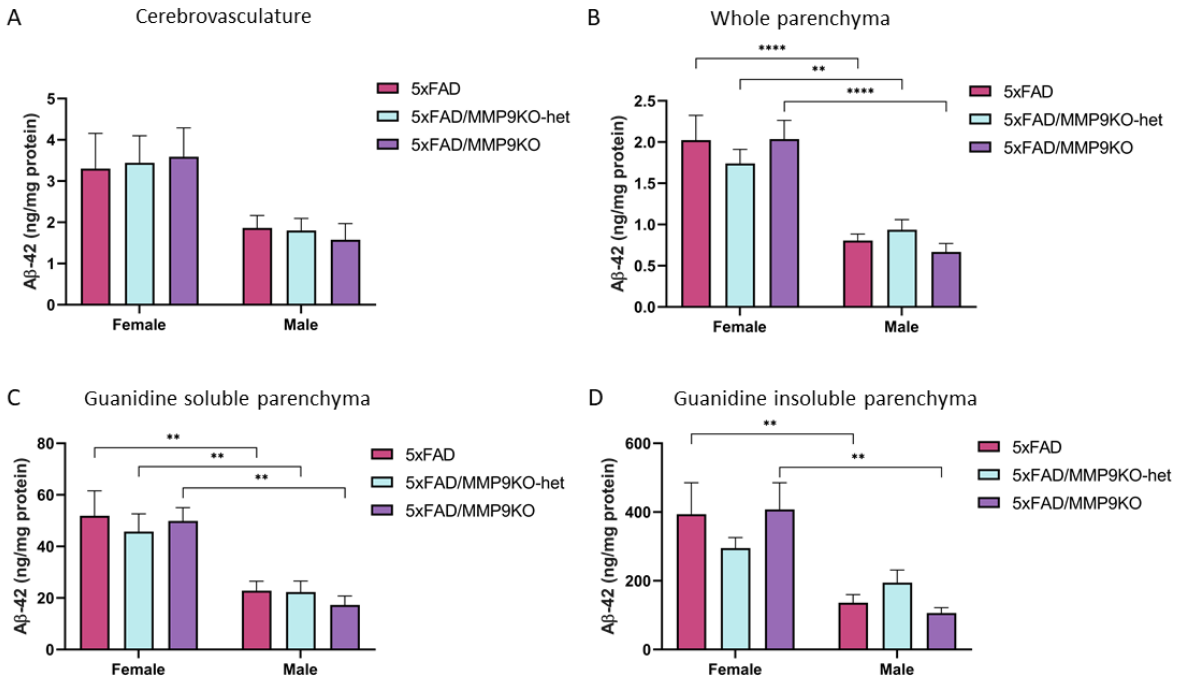

Figure 4.14: Analysis of Aβ-42 levels in the cerebrovasculature, whole parenchyma and the GS and GI parenchyma brain fractions.
Aβ-40 levels were examined in the (A) cerebrovasculature, (B) whole parenchyma, (C) GS parenchyma and (D) GI parenchyma of 5xFAD, 5xFAD/MMP9KO and 5xFAD/MMP9KO-het mice. Males and females were analysed separately due to large differences in amyloid levels. Values represent mean ± SEM (N = 6). Statistical significance was determined by two-way ANOVA followed by the BKY procedure. **p<0.01, ****p<0.0001.

4.3.2.5 LDLR and LRP1 levels

The cerebrovasculature of WT, 5xFAD, 5xFAD/MMP9KO, 5xFAD/MMP9KO-het and MMP9KO mice was analysed for LDLR and LRP1 levels to determine the effect of MMP-9 gene deletion on lipoprotein receptor shedding (Fig. 4.15b, d). No differences were detected between any genotype. Similarly, no differences were observed in receptor levels in the soluble brain fraction in any group (Fig. 4.15a, b). All genotypes displayed similar levels of LDLR and LRP1 in both brain fractions (Fig. 4.15). There were no gender differences observed in the analysis of LRP1 or LDLR.

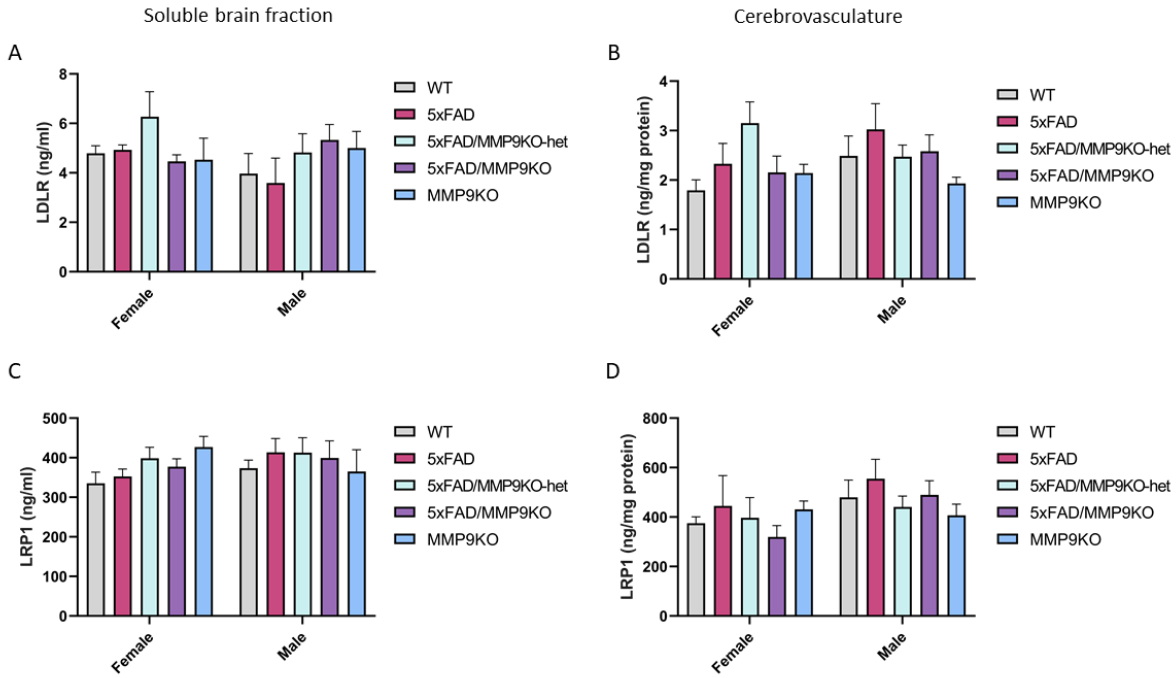

Figure 4.15: Analysis of LDLR and LRP1 levels in the cerebrovasculature and the soluble brain fraction.
Levels of the (A, B) LDLR receptor and (C, D) LRP1 receptor were analysed in the (A, C) soluble brain fraction and (B, D) the cerebrovasculature of wild type, 5xFAD, 5xFAD/MMP9KO, 5xFAD/MMP9KO-het and MMP9KO mice. Values represent mean ± SEM (N = 12). No statistically significant differences in LDLR or LRP1 levels were identified between any genotype in either brain fraction by a two-way ANOVA.

4.4 Discussion

Following the identification of several mechanisms by which apoE regulates MMP-9 function along with the reduced ability of the apoE4 isoform to attenuate MMP-9 levels and activity in AD, the effect of MMP-9 inhibition was assessed in E4FAD mice. Both MMP-9 and apoE4 have been implicated in early AD pathology, before the onset of cognitive impairment [99], [210], [211], [422], [423]. Therefore, in the current studies, treatment with the MMP-9 inhibitor, SB-3CT, began in 4-month-old E4FAD mice, an age at which these transgenic animals are in the early stages of plaque development [247]. The upregulation of MMP-9 in multiple degenerative and inflammatory disease states has prompted many to pursue therapies that reduce its detrimental effects. Increased MMP-9 levels are apparent in AD [200], [208], [309], along with other neurological diseases [203], [204], [227], [426]–[431], where they have been shown to perpetuate disease progression. Studies have shown that the benefits of MMP-9 inhibition following the injection of Aβ in rodents include improved cognition [212], [310]. In the present studies, the influence of MMP-9 in AD was examined by crossing 5xFAD mice with MMP9KO mice.

Behavioural and psychological symptoms are common in AD and can significantly impact social interactions, leading to social withdrawal [462]–[466]. The sociability of the mice used in the current studies was measured with the three-chamber test. While mice are generally social animals [454], the E4FAD mice did not display a preference for the mouse compared to the empty cage, indicating a reduced sociability. Similarly, though the 5xFAD mice did spend significantly more time with the mouse than the empty cage, it did so to a lesser degree than the WT mice, consistent with previous studies demonstrating reduced sociability in this mouse model progressing from 3 months of age [467]. This reduced interest in social interaction in 5xFAD mice was not apparent in the 5xFAD/MMP9KO mice, suggesting that removal of the MMP-9 gene in this AD mouse model beneficially impacted sociability. Furthermore, when a novel mouse was introduced, the WT, MMP9KO and the 5xFAD/MMP9KO mice all spent significantly more time with it

compared to the familiar mouse, however the 5xFAD mice did not, indicating an impairment in social recognition memory that has been previously reported for this AD mouse model [468]. Notably, this impairment was not observed when the MMP-9 gene was absent. The CA2 region of the hippocampus has been shown to be crucial for sociocognitive memory processing [469] and in 6-month-old 5xFAD mice, the hippocampus shows considerable amounts of Aβ [66]. Our previous research demonstrated that inhibition of MMP-9 mitigated the Aβ-induced lipoprotein receptor shedding in the brains of apoE4 mice in addition to increasing the clearance of intracranially injected Aβ from the brain to the periphery [222]. However, this does not appear to be the main mechanism underlying the improved social memory in the 5xFAD/MMP9KO mice since Aβ-40 and Aβ-42 levels in the parenchyma, cerebrovasculature and Aβ-40 levels in the plasma remained constant across genotypes. Moreover, the levels of the lipoprotein receptors were unchanged between the different genotypes, suggesting that there were no discernible effects on LRP1 and LDLR shedding. Although knocking out MMP-9 in the 5xFAD mice did not result in obvious shifts in Aβ, it may have prevented the involvement of MMP-9 in Aβ-induced cognitive deficits [212]. Mizoguchi et al. demonstrated that the injection of Aβ increases MMP-9 expression and that this increase is associated with the development of cognitive impairment and neurotoxicity [212]. Recognition memory, as measured by the novel object recognition test, was impaired in Aβ-injected WT mice but not MMP9KO mice or WT mice treated with an MMP inhibitor [212], coinciding with the current studies using the three-chamber test. Like other mouse models of AD, 5xFAD mice display increased levels of MMP-9 compared to WT mice [445], [446], which could be contributing to the observed deficits in social recognition memory in these mice. Thus MMP-9 could be a target to improve social memory.

While it was anticipated that MMP-9 modulation would alter brain Aβ levels by facilitating lipoprotein receptor transit across the BBB, removing MMP-9 may prevent other harmful actions of the enzyme, and could explain the differences observed between the 5xFAD and the 5xFAD/MMP9KO mice. For instance, the degradation of matrix proteins in the vascular basal

lamina by MMP-9, leading to the disruption of the BBB, has been suggested to contribute to the brain damage that occurs following cerebral ischemia [470]–[472], which has been shown to be attenuated by MMP-9 inhibition [166], [432]. Alternatively, the degradation of laminin or other matrix proteins by MMP-9 throughout the brain may disrupt cell–matrix interactions and play a role in neuronal cell death [206], which may be reduced following the deletion of MMP-9. In fact, inhibition of MMP-9 has been shown to reduce tissue damage, neutrophil infiltration, oxidative stress and degenerating neurons [166], [431], [432], all factors which could contribute to the impaired social memory in the 5xFAD mice [236], [240], [473]–[476].

MMPs, in particular MMP-9, have been suggested to have a generalised role in the maintenance of long-term potentiation (LTP) and the nonpathological synaptic plasticity and function in intact adult brains [477]–[480]. In previous studies MMP-9 inhibition has led to the disruption of late-phase LTP [477]–[480]. However, excessive MMP-9 activity is deleterious to the cells and MMP-9 inhibition has also been shown to enhance LTP under conditions of excessive and prolonged MMP-9 activity [481]. Under normal conditions MMP-9 activity is strictly regulated, and the transient proteolytic activity required for structural remodelling is focal and quickly terminated following its completion. Conversely, in pathological conditions, MMP-9 activity is widespread and sustained, leading to abnormal synaptic plasticity and impairments in cognitive function [481], [482], which may describe the deficits in social recognition memory observed in the 5xFAD but not the 5xFAD/MMP9KO mice.

The modulation of MMP-9 in 5xFAD mice appeared to be more effective in male mice, which may be because they have inherently less pathology compared to female mice [236], [239]. This is consistent with the results from clinical studies, where treatments are most effective in the early stages of pathology [298], [299]. In contrast to social recognition memory, spatial recognition memory was not impacted by MMP-9 deletion or inhibition in the mice, however, male E4FAD mice treated with SB-3CT located the hidden platform slightly faster than the vehicle-treated mice

over the course of the 5 days. This finding was not statistically significant, possibly owing to the reduced number of mice in this subgroup analysis (N=5) [483], [484]. Spatial disorientation is frequently observed in AD with patients displaying impaired visuospatial memory [485]–[487]. Spatial recognition memory has been previously shown to be impaired in 4-month-old E4FAD mice using the two-trial Y-maze and the Morris Water Maze [251], however, in the RAWM used in these studies, the control E4FAD mice continued to learn each day, making few mistakes by day 5 and finding the platform quickly. Likewise, the performance of the 5xFAD mice at 6 months of age was similar to the WT mice and did not exhibit deficits in spatial memory. Deficits in spatial memory and recognition memory are both initial symptoms of AD [488], though spatial memory typically presents earlier in the spectrum of cognitive impairment than recognition memory [468], [489]–[492]. In contrast, in the current studies, the 6-month-old 5xFAD mice displayed impairment of social recognition memory but not spatial memory. While memory impairment in this 5xFAD mouse strain has been detected as young as 1 month of age through the Morris water maze [493], [494], others have found that 5xFAD mice display normal spatial memory function in this test until 7 months of age [238] or even up to 12 months of age [495]. Likewise, recognition memory has been shown to emerge at 4 months of age [495] in one study and at 9 months of age in another [468]. These results indicate that behaviour results can vary considerably by experiment and memory impairment can be specific to context, modality, and/or environment [467]. Because the E4FAD and the 5xFAD made few errors by day 5, it is difficult to discern whether the SB-3CT treatment or the MMP-9 knockout had any impact on spatial memory in these studies. Future studies would need to assess the effect of both approaches in older mice with more apparent deficits in spatial memory, whilst taking into account potential differences in gender.

It has been demonstrated that 5xFAD mice display reduced anxiety in the EPM compared to WT mice, as measured by increased time spent in the open arms compared to the closed arms [240], [496], [497], which coincides with the results from the present studies and reflects a tendency toward disinhibition. This disinhibition is one of the neuropsychiatric symptoms seen in AD

patients, manifesting as impulsive behaviour and the disregard for danger [498]–[500]. The preference for the open arms in the EPM by 5xFAD mice has been suggested to be caused by impaired tactile sensation from the vibrissae, which is thought to lead to overstimulation and consequent avoidance of the closed arms [468]. However, the 5xFAD mice also spent less time in the outer edges of the OFT where this factor has less influence. Conversely, the behaviour of the 5xFAD/MMP9KO mice in the OFT and the EPM was similar to the WT mice, indicating that removing MMP-9 may have influenced anxiolytic behaviour in the 5xFAD mice. It is necessary to analyse locomotor behaviour of the mice in these tests since anxiety measures can be confounded by levels of activity [501]. The increased locomotor activity in the 5xFADs has also been previously documented and attributed to alterations in neurotransmitter levels [468], [502] in addition to decreased anxiety [503]. In the present studies 5xFAD/MMP9KO mice had significantly lower locomotor activity compared to the 5xFAD mice as measured by distanced moved and average velocity, suggesting that the MMP-9 gene may be influencing anxiety-related behaviours. MMP-9 has previously been implicated in an increased susceptibility to anxiety disorders [504] as well as having elevated levels in other psychiatric illnesses [505]–[507]. Human genomics and proteomics studies have revealed that aberrant inflammatory responses, gene regulation and synaptic plasticity are the major players underlying neuropsychiatric disorders. Since MMP-9 has been implicated in both the inflammatory response and synaptic plasticity, and is tightly controlled by gene regulation, it may contribute to the development of psychiatric symptoms [507]. However, more research needs to be conducted to understand the precise mechanisms involved.

The altered anxiety in E4FAD mice indicates that treatment with SB-3CT is having an effect, however due to limitations in the detection methods, the proteolytic activity of MMP-9 in the brain could not be measured. This in vivo finding is consistent with other reports owing to the rapid degradation of the activated MMP-9 enzyme [206]. The mechanism of action of the SB-3CT compound is to inhibit the activity of MMP-9, not the expression [508] and thus total MMP-9 levels in the brains of the E4FAD mice remained unaltered. Therefore, while seven days of SB-

3CT treatment through intraperitoneal injections has been previously shown to effectively inhibit MMP-9 [204], [436], it is not certain whether the observed effect on anxiety in the current studies was caused by the inhibition of MMP-9 activity or another effect of the drug.

The inherent limitations of transgenic AD mouse models when investigating the underlying mechanisms and potential treatments for sporadic AD must be noted. 5xFAD mice are based on EOAD and co-express five FAD mutations, resulting in excessively high levels of Aβ-42, and therefore is not fully equipped to study the progression of sporadic AD, which represents >97% of the AD population [509], [510]. The inability to recapitulate sporadic AD in animal models due to the complex and heterogenous nature of the disease has hindered the development of suitable treatments. However, by combining common risk alleles, researchers are seeking to develop more translationally relevant mouse models that better reflect this more prevalent form of AD [509], [510]. One example of this is a new mouse strain that has been created by the MODEL-AD consortium which expresses two of the strongest genetic risk factors for LOAD, APOE and TREM2, alongside human Aβ [511], [512]. Additional LOAD risk alleles along with environmental risk factors will be added to this model in order to generate a phenotype which more closely aligns with that of sporadic AD [511], [512]. Furthermore, since AD is primarily an age-related disorder, a translatable model of the disease would need to include an age component to fully replicate the human AD condition. The use of more accurate animal models of LOAD would facilitate the testing of potential treatments and may result in different outcomes in preclinical studies owing to the different causes and progression of sporadic AD compared to EOAD. With respect to the current studies, using mouse models of sporadic AD would give a better indication of the effect of MMP-9 inhibition on the underlying mechanisms and the cognitive symptoms of AD, and its potential as a therapeutic target for this disorder.

Collectively, the results from this Chapter indicate that reducing aberrant MMP-9 activity or expression has beneficial effects in transgenic AD mouse models, in particular on sociability, social

recognition memory and reduced anxiety disinhibition. Removing the MMP-9 gene in 5xFAD mice resulted in more apparent differences compared to pharmacological inhibition in E4FAD mice, which may be due to several factors. For instance, the increased length of time and earlier commencement (from birth) of impeding MMP-9's harmful effects, and the lack of involvement of the APOE4 isoform may all contribute to the greater improvement in behaviour seen in the 5xFAD/MMP9KO mice compared to the SB-3CT-treated E4FAD mice. Our prior work [222], [319] and the data presented in previous chapters appeared to indicate MMP-9 levels were elevated and harmful in AD, particularly when associated with the APOE4 isoform, and that MMP-9 modulation would promote Aβ elimination from the brain. While this appears not to be the case in the current AD animal studies, MMP-9 did impact behaviour but seemingly through another mechanism. The results from these studies offer further evidence for an important role of MMP-9 in the development of cognitive impairment and the AD phenotype. Inhibiting the harmful actions of MMP-9 may represent a promising therapeutic strategy for the treatment of AD and other neurological disorders.

Chapter 5: Conclusions

Earlier work conducted by our group demonstrated that apoE influences the clearance of Aβ through the BBB in an isoform dependent manner by impacting the ectodomain shedding of LRP1 and LDLR [182]. Our team found this regulation involved MMP-9, an enzyme implicated in AD pathology. MMP-9 treatment in brain endothelial cells and freshly isolated mouse cerebral vessels dose-dependently increased lipoprotein receptor shedding [222]. Furthermore, treatment with the MMP-9 inhibitor, SB-3CT, increased Aβ transit across the BBB in an *in vitro* model, and enhanced the clearance of intracranially injected Aβ from the brain to the periphery in apoE4-TR mice [222]. Therefore, our team hypothesised that apoE was influencing Aβ transit across the BBB through the regulation of MMP-9 and its ability to elicit lipoprotein receptor shedding.

In this thesis, apoE was shown to modulate several regulatory mechanisms of MMP-9 in an apoE isoform-specific manner. A clear indication of this regulation was shown in Chapter 3, where apoE dose-dependently inhibited the activity of MMP-9 in a cell-free assay. ApoE2 showed the greatest inhibition of MMP-9 activity, while apoE4 displayed the weakest inhibition. Owing to the cell-free nature of this assay, it is plausible that apoE is directly interacting with MMP-9 to produce these effects. This theory is supported by the studies using biolayer interferometry, in which it is illustrated that recombinant apoE can bind to recombinant MMP-9 and that the apoE4 isoform demonstrated the weakest binding affinity. Furthermore, confocal microscopic analysis in EFAD animals showed that apoE and MMP-9 were associated within the same areas of brain endothelial cells, further alluding to an interaction between these two molecules within the brain.

Notably, APOE isoform-specific differences in MMP-9 levels were detected in both human and mouse AD brain samples and these effects were more apparent upon insult, i.e. the presence of AD (APOE2<APOE3<APOE4). The results of these studies suggest that the combination of AD and APOE4 results in a greater elevation of MMP-9 levels in the brain. Deposition of Aβ in the AD brain has been reported to be higher in APOE4 carriers [131], [132] and our group and

others have shown that Aβ induces MMP-9 expression [212], [222], [343]. The combination of elevated MMP-9 levels and the APOE4 isoform could exacerbate the development of AD pathology through a vicious circle, where both restrict the clearance of Aβ from the brain to the periphery, contributing to the accumulation of Aβ in the brain, which in turn promotes further expression of MMP-9.

In looking more broadly at the data in Chapters 2 and 3, MMP-9 cellular secretion in the presence of apoE2 and cerebrovascular MMP-9 levels in the E2FAD mice were substantially lower than the other apoE isoforms (apoE2<<apoE3=apoE4). The effect of apoE2 on cellular secretion and MMP-9 levels may be similar to that reported for the secretion of Aβ, where apoE2 significantly attenuated the production of APP and secretion of Aβ compared to the other apoE isoforms [347]. Conversely, the results from the MMP-9 activity and binding studies indicate apoE4 may be most relevant in the regulation of MMP-9 function. The binding affinity of apoE4 to MMP-9 and the influence of apoE4 on MMP-9 activity were considerably lower than the other isoforms (apoE2=apoE3>>apoE4). In total, while apoE can impact MMP-9 processing overall, the individual isoforms appear to do so through different mechanisms as our findings suggest apoE2 is most effective in suppressing the production and/or cellular secretion of MMP-9, while apoE4 is least effective in binding MMP-9 and modulating its activity [319].

Both MMP-9 and APOE4 have been implicated in early AD pathology, before the onset of cognitive impairment [99], [210], [211], [422], [423]. Therefore, therapeutic approaches targeting MMP-9 may require early administration to see the greatest benefits. SB-3CT has been shown to have beneficial effects in many neurological disorders including ischemia, TBI, stroke, subarachnoid haemorrhage and spinal cord injury [436], [441], [442], [444], [513] and in primary human pericyte cultures following the administration of toxic oligomeric Aβ [362]. In the current studies, while 30 days of SB-3CT treatment in E4FAD mice showed reduced anxiety disinhibition, no effects on cognition or amyloid pathology were observed. Owing to the limitations encountered

in detecting active MMP-9 in the mouse brains, it is uncertain how much MMP-9 activity was inhibited in the treated mice. This would need to be rectified by using a more sensitive method to detect the active species of MMP-9, which is short lived and typically low in concentration [206]. Alternatively, to indirectly gauge the overall activity of MMP-9 in SB-3CT and vehicle-treated mouse brains, levels of MMP-9 substrates in the brain could be measured. In prior reporting, this dose of SB-3CT showed significant reductions in MMP-9 activity in the brain using a treatment paradigm more acute than that used in the present studies [436]. Thus, it seems likely MMP-9 was inhibited in the current studies, but a more chronic treatment paradigm may be necessary to overcome the AD phenotype, since SB-3CT treatment began in E4FAD mice at 4-months of age, at which time pathology is already apparent [237], [238].

In providing a more definitive method to investigate the role of MMP-9 in AD animals, a mouse model was created in which the MMP-9 gene was genetically removed in 5xFAD mice. Behaviour changes in these mice compared to the 5xFAD mice were more evident than the pharmacological inhibition studies. Notably, the absence of the MMP-9 gene in 5xFAD mice protected them against deficits in sociability and social recognition memory and restored anxiety disinhibition. While our prior work indicated that MMP-9 modulation may be beneficial due to the enhanced elimination of Aβ through the BBB [222], the data presented in Chapter 4 suggests that MMP-9 modulation did impact behaviour, but via another mechanism, as Aβ levels in the brain and plasma were unchanged. MMP-9 has been shown to be upregulated in 5xFAD mice compared to WT mice, and has been suggested to contribute, at least in part, to the detrimental effects observed in this mouse model including those relating to neuroinflammation and neurotoxicity [445], [446]. In that regard, the inhibition of MMP-9 in these mice may act to combat and reverse some of these effects, leading to memory improvement.

As described in Chapter 1.4, to date, there is no disease-modifying therapy for AD patients, as the approved treatments merely treat symptoms of the disease instead of altering disease progression

and preventing cognitive decline. While these current drugs may provide transient benefits to patients by enhancing their cognitive abilities, this does not translate to long lasting improvements. Hence, disease modifying treatments are required for the successful treatment of AD. The investigation and advancement of alternative therapies to the ones currently used in the clinic remains an essential part of drug development. As indicated by the recent failures of amyloid-directed therapies in the clinic, targeting Aβ may not be enough to fully resolve AD [514]. MMP-9 inhibitors represent one therapeutic avenue that may help to alleviate behavioural deficits and restore normal brain function. As discussed in Chapter 4, this could potentially occur through an amyloid-independent mechanism. Due to the complex and diverse nature of AD pathology, inhibition of MMP-9 may be more beneficial in some individuals than others. In this regard, the inhibition of MMP-9 in APOE4 carriers may confer additional positive effects, owing to the higher levels and inefficient modulation of MMP-9 exhibited by this genotype.

Initial trials with MMP inhibitors for the treatment of cancer yielded negative results, resulting in undesirable side effects such as musculoskeletal syndrome [515]–[517]. This was later found to be due to the broad-spectrum nature of these drugs and that inhibition of certain MMPs (MMP-2, MMP-9, MMP-13 and MT1-MMP) did not result in these adverse effects [518], [519]. Currently, there is one MMP inhibitor, doxycycline, which is FDA approved for periodontal disease treatment [515], [520] and there are other non-specific MMP inhibitors being tested in clinical trials as potential cancer treatments [201], [202]. The most recent generation of MMP inhibitors are more specific and display better pharmacokinetics resulting in less toxicity [515]. One such compound that has been recently discovered, JNJ0966, has been shown to be highly selective in its inhibition of MMP-9, and does not inhibit MMP-2, which is closely related [375]. These compounds offer renewed interest in MMP-9 inhibitors and their potential in AD and other disorders [515].

The data presented in Chapter 4 suggest that inhibition of MMP-9 in AD can lead to positive behaviour changes including the rescue of social recognition memory. Additionally, the studies

discussed in Chapter 1.2.3 signal that the inhibition of aberrant elevated MMP-9 expression and activity may be beneficial in numerous other diseases [194], [199], [200], [203], [204], [227], [426]–[431]. The combined results from these studies suggest that inhibiting MMP-9 can lead to a wide array of effects including, for example, the attenuation of the immune response and the subsequent tissue damage in inflammatory conditions [199], [200], improvement of cardiac function [199] and the reduction of BBB dysfunction [203], neuronal apoptosis [204], epileptogenesis [426], myelin destruction [429], [430], cytokine activation [430], oxidative stress, oedema and neurodegeneration [431]. Consequently, the continued exploration of MMP-9 inhibitors as therapeutic agents may have broader applications than in AD alone. Likewise, as APOE genotype has been shown to influence the progression of many of these diseases [521]–[523], it follows that there may be interaction between apoE and MMP-9 in other disorders in which the inhibition of MMP-9 may be of a higher value in APOE4 carriers.

APOE isoforms are a major genetic determinant of developing sporadic AD [109], [110], and are therefore undisputedly a prominent target in the advancement of therapies to combat AD. Hence it is crucial to understand the mechanisms involved in the development of cognitive impairment and the AD phenotype in depth, in particular with respect to APOE genotype, in order to formulate potential therapies [308]. In conclusion, the data presented in this thesis support a role of apoE in the modulation of MMP-9 disposition, particularly upon insult, i.e. the AD environment, and that apoE isoforms differ in their ability to regulate MMP-9 function (apoE2>apoE3>apoE4). Furthermore, the modulation of elevated MMP-9 expression and activity may be a promising therapeutic approach for AD and potentially other neurological disorders with aberrant MMP-9 activity and underlying behavioural deficits.

Chapter 6: Future directions

In Chapter 3 of this thesis the binding affinities of each recombinant apoE isoform with MMP-9 were investigated. However, due to the presence of potentially confounding proteins in the conditioned media containing glia-lipidated apoE, the binding affinities of these apoE species with MMP-9 could not be assessed. Considering that this source of apoE is more physiologically relevant than the delipidated recombinant apoE or the artificially lipidated apoE, it would be advantageous to determine whether the binding pattern follows that seen with recombinant apoE in the current studies (apoE2=apoE3>apoE4). Future studies would require a larger amount of the glia-conditioned apoE, or another source of physiologically lipidated apoE, so that it can be isolated and purified before analysis. For example, immortalised cell lines from primary glial cultures derived from apoE-TR mice could be used to generate large quantities of apoE-containing lipoproteins [400].

Further analysis of the *in vivo* samples will elucidate the impact of MMP-9 gene removal in the 5xFAD mice. Brain hemispheres were collected from these mice and fixed in 4% PFA for tissue processing and extended investigation using immunofluorescence. This will help ascertain any pathological changes in the 5xFAD/MMP9KO mice which may be underlying the observed behavioural differences. As noted previously, this may involve changes in neurodegeneration, oxidative stress or neuroinflammation [166], [431], [445], [446]. The studies conducted by Mizoguchi et al., point to the direct involvement of MMP-9 in Aβ-induced cognitive deficits, since MMP-9 inhibition and knockout prevented the impairment of social recognition memory [524]. Therefore, a more detailed analysis of specific changes in the Aβ population following MMP-9 removal, for instance shifts in the brain location, forms, or toxic species of Aβ, may be necessary to understand the influence of MMP-9 in these mechanisms.

These studies with the MMP9KO mice provide a simplified view of the deleterious actions of MMP-9 in AD and the benefits associated with inhibiting it, without the complexity of apoE

isoforms. However, apoE-driven AD pathologies differ from pure amyloid pathologies [525], [526] and, as detailed in this thesis, apoE can modulate multiple aspects of MMP-9 regulation other than elevated expression and may still influence MMP-9 disposition after its induction. Hence, it would be interesting to build on these *in vivo* studies and cross MMP9KO mice with EFAD mice, to generate EFAD mice in which the MMP-9 gene is deleted. Another possibility would be to investigate the conditional knockout of MMP-9 in specific cells, for instance endothelial cells, to establish whether the release of MMP-9 in certain areas of the brain are more damaging. These experiments would examine the behaviour (spatial memory, learning, anxiety, and social memory), in addition to AD-related pathological changes on a molecular level, including Aβ disposition, using different detection methods such as ELISA, immunoblotting, and immunofluorescence microscopy. This study will provide us with more detailed information about the interactions between APOE and MMP-9 and the benefit of inhibiting MMP-9 in APOE4 carriers in particular, who represent a large proportion of AD patients.

Chapter 7: References

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